At the Crossroad of DNA Damage Checkpoint and DNA Repair: Physical and Functional Interactions between Checkpoint Sensors and Components of the Base Excision Repair Machinery

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TABLE OF CONTENTS

2. SUMMARY

Maintenance of genomic stability relies on the accurate duplication of the genome and the continuous monitoring of its integrity. In the response to DNA damage and replication stress cells activate checkpoint signaling pathways that halt cell cycle progression, induce DNA repair or trigger apoptosis. In mammalian cells several proteins are involved in damage sensing and in activation of the checkpoint signaling cascade. Among them are the ATM and ATR proteins, which belong to the phosphatidylinositide kinase-like kinase family, the Rad17-replication factor C (Rad17-RF-C_{2-5}) and the Rad9-Rad1-Hus1 (9-1-1) checkpoint complexes, which have structural similarities to the replication clamp loader, RF-C and the replication clamp, proliferating cell nuclear antigen (PCNA), respectively, To ensure proper removal of the damage, the checkpoint response must be finely coordinated with DNA repair. Therefore, the checkpoints are proposed to act not simply as a switch for arrest, but also directly participate in the DNA repair machinery. The major repair pathway protecting cells against a single-base DNA damage is base excision repair (BER). Two BER sub-pathways have been identified, and have been classified according to the length of the repair patch as either 'short-patch' BER (SP-BER; one nucleotide) or 'long-patch' BER (LP-BER; more than one nucleotide). In the minimal process, LP-BER consists of five steps carried out by different proteins. First, the damage base is identified and removed from the DNA helix by the action of specific DNA glycosylase. Second, the resulting abasic site is recognized by apurinic/apyrimidinic endonuclease 1 (APE1), which incises the damaged strand, Third, DNA polymerase (Pol) β fills in the gap using complementary strand synthesis and displaces up to 10 nucleotides of downstream sequence. The resulting flap is then, fourth, removed by the action of Flap endonuclease 1 (Fen 1). Fifth, the remaining nick is sealed by DNA ligase I (Lig I). Recent studies have demonstrated that the 9-1-1 complex, besides its functions in DNA damage sensing and signaling pathways, plays also a direct role in various DNA repair pathways. Recent investigations indicated that the 9-1-1 clamp interacts and/or stimulates several BER components namely Pol β, Fen 1, Lig I and the MutY homologue of *Schizosaccharomyces pombe.*

The aim of the thesis was to identify novel interactions that occur between the components of the LP-BER machinery and the two DNA damage sensors, the 9-1-1 complex and the Rad17-RF-C_{2-5} complex. In the first part of the work, the interactions between APE1 and the 9-1-1 complex were studied. The 9-1-1 complex was shown to interact with APE1 *in vitro* and specifically stimulated the endonuclease activity of APE1. Co-immunoprecipitation experiments using total cell lysates prepared from untreated, human 293T cells confirmed that the two protein interact *in vivo*. In response to genotoxic stress, the 9-1-1 complex and APE1 co-localized at the sites of DNA damage, suggesting that the checkpoint clamp might stimulate there the endonuclease activity of APE 1 in BER. Moreover, The 9-1-1 complex stimulated a reconstituted LP-BER system *in vitro* and this was due to the effect the 9-1-1 complex exerted on the endonuclease activity of APE1 and the strand displacement activity of Pol β. Importantly, under conditions applied in the reconstituted LP-BER assay, no effect of the 9-1-1 complex on the enzymatic activities of Fen 1 and Lig I was observed leading to the conclusion that in the reconstituted LP-BER *in vitro* system, a hierarchy of interactions between the 9-1-1 complex and the components of the LP-BER machinery exists.

In the second part of the thesis the novel interactions between APE1 and the checkpoint clamp loader, Rad17-RF-C_{2-5} complex are described. APE1 was shown to interact with the Rad17- RF-C_{2-5} complex in *vivo* and *in vitro* as revealed by immunoprecipitation experiments using human, total cell extracts and pulldown assays with purified proteins. Moreover, APE1 and Rad17 co-localized to the same nuclear foci in human cells and this co-localization was enhanced upon DNA damage.

In summary, these data suggest that the checkpoint clamp/clamp loader complexes besides their functions in DNA damage sensing and signaling pathways, can directly participate in DNA repair thus providing a link between DNA repair and DNA damage checkpoints.

3. ZUSAMMENFASSUNG

Die Erhaltung der genomischen Stabilität bedarf der korrekten Verdoppelung des Genoms, sowie die ständige Überwachung von dessen Integrität. Als Antwort auf DNA-Schäden und Replikationsstress aktivieren Zellen den sogenannten Kontrollpunkt-Signalisierungsweg (=Checkpoint signaling pathway), welcher den Fortlauf des Zellzyklus blockiert, sowie die DNA-Reparatur reguliert und nötigenfalls den programmielten Zelltod (Apoptosis) einleitet. In Säugetierzellen sind es die ATM und die ATR Proteine, welche zur phosphatidylinositid Kinase-ähnlichen Familie gehören, sowie der Rad17-Replikationsfaktor C (Rad17-RF-C_{2-5}) und der Rad9-Rad1-Hus1 (9-1-1) Checkpointproteinkomplex. Diese weisen strukturelle Ähnlichkeit auf mit RF-C und PCNA auf, welche beide in die Schadenserkennung involviert sind. Um sicherzustellen, dass der Schaden korrekt entfernt wird, muss der Kontrollpunkt-Signalisierungsweg mit der DNA-Reparatur fein koordiniert werden. Aus diesem Grund wird angenommen, dass die Kontrollpunkte nicht nur den Zellzyklus blockieren, sondern auch direkt an der DNA-Reparatur beteiligt sind.

Der Hauptreparaturweg, den die Zelle gegen einen einzelnen Basenschaden schütz, ist der Base-Entfernungsreparaturweg (Base Excision Repair = BER). Dabei konnten mittels *in vitro* Methoden zwei Untergruppen unterschieden werden, nämlich der Kurzstrecken-BER Weg (Short Patch Base Excision Repair = SP-BER), sowie der Langstrecken-BER Weg (Long Patch Base Excision Repair = LP-BER), welche sich in der Länge des ersetzten DNA-Stückes unterscheiden (SP-BER ersetzt 1 Nukleotid, der LP-BER ersetzt 2- 10 Nukleotide). Im einfachsten Fall besteht der LP-BER aus fünf Schritten, welche von fünf verschiedenen Proteinen ausgeführt werden. Als erstes wird die beschädigte Base erkannt und aus der DNA-Helix entfernt, und zwar durch die Aktivität spezifischer DNA Glycosylasen. Die daraus resultierende abasische Stelle wird von der Apurinischen/Apyrimidinischen Endonuklease 1 (APE1) erkannt, welche dann den beschädigten DNA-Strang schneidet. Als nächstes füllt die DNA-Polymerase (Pol) β die fehlende Stelle wieder auf, und ersetzt bis zu 10 Nukleotide der abwärtsliegenden Sequenz, indem sie den komplementären, unbeschädigten Strang als Matrize benutzt. Der dadurch entstandene Überhang wird dann von der Flap Endonuklease 1 (Fen 1) beseitigt,

was zu einer Kerbe im reparierten DNA-Strang führt, welche schliesslich von der DNA Ligase I (Lig I) verknüpft wird. Kürzlich durchgeführte Studien haben gezeigt, dass der 9-1-1 Komplex neben seiner Funktion in der DNA-Schadenserkennung und Signalisierung auch eine direkte Rolle in den verschiedenen DNA-Reparaturwegen spielt. Jüngste Untersuchungen deuten an, dass der 9-1-1 Komplex mit verschiedenen BER-Proteinen, wie Pol β, Fen 1, Lig I und dem MutY Homolog von *Schizosaccharomyces pombe* interagiert und diese stimuliert.

Das Ziel der vorliegenden Dissertation war es, neue Interaktionen zu identifizieren, welche zwischen den Komponenten der BER-Maschinerie, sowie den zwei DNA-Schadenssensorenkomplexen, 9-1-1 und Rad17-RF-C_{2-5}, stattfinden. Im ersten Teil der Arbeit wurden die Interaktionen zwischen APE1 und dem 9-1-1 Komplex genauer studiert. Es konnte gezeigt werden, dass der 9-1-1 Komplex *in vitro* tatsächlich mit dem APE1 Protein interagiert und auch dessen Endonuclease-Aktivität stimuliert. Mittels Co-Immunoprezipitationsexperimenten mit Zellextrakt von unbehandelten menschlichen 293T Zellen konnte auch die Interaktion der zwei Proteine *in vivo* bestätigt werden. Als Antwort auf genotoxischen Stress co-lokalisierten der 9-1-1 Komplex und APE1 an den Stellen des DNA-Schadens, wo dann der 9-1-1 Komplex die Endonuclease-Aktivität von APE1 im BER weiter stimulieren konnte. Ferner konnte der 9-1-1 Komplex ein *in vitro* nachgestelltes LP-BER System stimulieren, wobei diese vor allem auf den Effekt, den der 9-1-1 Komplex auf APE1 und die Strangaustauschaktivität von Pol β hatte, zurückgeführt werden konnte. Interessanterweise konnte unter den verwendeten Bedingungen kein Effekt vom 9-1-1 Komplex auf die enzymatische Aktivität von Fen 1 und Lig I beobachtet werden, was zur Schlussfolgerung führte, dass im verwendeten nachgestellten *in vitro* LP-BER System eine Hierarchie der Interaktionen zwischen dem 9-1-1 Komplex sowie den Komponenten der LP-BER Maschinerie existiert.

Im zweiten Teil der Arbeit wurde die neue Interaktion zwischen APE1 und dem Kontrollpunkt-Klammerlader, Rad17-RF-C_{2-5} Komplex, genauer untersucht. Es konnte mit Hilfe von Immunoprezipitationsexperimenten mit menschlichen totalen Zellextrakten und mit Pulldown Experimenten mit gereinigten Proteinen gezeigt werden, dass APE1 mit dem Rad17-RF-C_{2-5} Komplex sowohl *in vitro* als auch *in vivo*

interagiert. Zusätzlich co-lokalisierten APE1 und Rad17 in denselben nukleären Foci, was verstärkt nach DNA-Schädigung beobachtet werden konnte.

Insgesammt deuten diese Daten darauf hin, dass die Kontrollpunktklammer-/Klammerlader-Komplexe neben ihrer Funktion bei der DNA-Schadensaufspürung und -signalisierung auch ganz direkt in der DNA Reparatur mitwirken und somit eine direkte Querverbindung herstellen zwischen DNA Reparatur und DNA Schadenskontrollpunkten.

4. ABBREAVIATIONS

aa	amino acid
Amp	ampicillin
APE1	Apurinic/apyrimidinic endonuclease 1
ATM	ataxia telangiectasia mutated kinase
ATP	adenosine 5'-triphosphate
ATR	ATM and Rad3-related kinase
ATRIP	ATR-interacting protein
BER	Base Excision Repair
bp	base pair(s)
BSA	bovine serum albumin
°C	degree Celsius
Ci	curie
DNA	deoxyribonucleic acid
ds	double stranded
DTT	dithiothreitol
E. coli	Escherichia coli
EDTA	ethylenediamine tetraacetic acid
Fen 1	flap endonuclease 1
FPLC	fast pressure liquid chromatography
g	acceleration due to gravity
g	gram
IR	Ionizing radiation

h	human
His	histidine
H_2O_2	Hydrogen peroxidase
kb	kilo base(s)
kDa	kilo Dalton
Lig I	DNA ligase I
M	molar (concentration)
2-ME	2-mercaptoethanol
mg	milligram(s)
μg	microgram(s)
min	minute(s)
MMS	methyl methane sulphonate
mol	mole(s)
μmol	micromole(s)
M_r	relative molecular mass
ng	nanogram(s)
NP-40	Nonidet P40
PAGE	polyacrylamide gel electrophoresis
PCNA	proliferating cell nuclear antigen
PiKKs	phosphoinositide 3-kinase-related kinases
pmol	picomole(s)
PMSF	phenyl- methylsulfonyl fluoride (serine -protease inhibito
Pol	DNA polymerase
RF-C	replication factor C

RNA	ribonucleic acid
RP-A	replication protein A
rpm	rounds per minute
RT	room temperature
SDS	sodium dodecyl sulfate
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophores
ss	single stranded
The 9-1-1 complex	The Rad9-Rad1-Hus1 complex
The Rad17-RF-C_{2-5} comple›	The Rad17-Replication Factor-C_{2-5} complex
Tris	Tris-(hydroxymethyl)-aminoethane
UV	ultra violet
(v/v)	volume per volume

5. INTRODUCTION AND REVIEW ARTICLE

5.1. DNA damage response mechanisms in the mammalian cell

The ability to accurately duplicate the genome and correctly segregate damage-free chromosomes to daughter cells is crucial to for the health and longevity of all organisms. To ensure the fidelity of these processes, different cellular responses are induced that enable the cell either to eliminate or handle the damage. In case of an extensive damage the apoptosis in activated. These DNA damage response reactions include: removal of DNA damage and restoration of the continuity of the DNA duplex; activation of a DNA damage checkpoint, which arrests cell cycle progression in order to allow repair and prevent the transmission of damaged or incompletely replicated chromosomes; transcriptional response, which causes changes in the transcription profile that may be beneficial to the cell and apoptosis, which finally eliminates heavily damaged or seriously deregulated cells (1). DNA repair mechanisms include base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), the double-strand break repair (DSBR) pathways homologous recombination repair (HR) and non-homologous end joining (NHEJ) and cross-link repair. The DNA damage checkpoints employ damage sensor proteins, such as ATM, ATR, the Rad17-RF-C_{2-5} complex, and the 9-1-1 complex, to detect DNA damage and to initiate signal transduction cascades that employ Chk1 and Chk2 Ser/Thr kinases and Cdc25 phosphatases. The signal transducers activate p53 and inactivate cyclin-dependent kinases to inhibit cell cycle progression from G1 to S (the G1/S checkpoint), DNA replication (the intra-S checkpoint), or G2 to mitosis (the G2/M checkpoint), promote DNA repair and/or induce apoptosis. However, to ensure the proper removal of the damage and prevent genomic instability, a clear cross-talk between different response pathways must occur. Indeed, many proteins which functions are primary involved in one response may participate in other responses as well, thus providing molecular links that connect different DNA damage responses.

5.2. The checkpoint clamp, Rad9 –Rad1-Hus1 complex

5.2.1. General overview

The *Rad9*, *Hus1*, and *Rad1* orthologs (using *Schizosaccharomyces pombe* nomenclature) were initially found in genetic screens designed to identify genes that affected sensitivity to genotoxic substances. Although required for activation of checkpoint signaling pathways, the initial cloning of these genes did not provide a clear answer about the functions of the encoded proteins, and how exactly they participate in checkpoint activation (2). First insights about the roles of these proteins came from studies using *in silico* molecular modeling techniques, which predicted that Hus1, Rad1, and Rad9 orthologs structurally resembled proliferating nuclear antigen (PCNA), a doughnut-shaped homotrimeric complex (3-11). PCNA is a sliding clamp that is loaded around DNA at the sites of ongoing DNA replication where it serves as a sliding platform that tethers replication proteins to the DNA. In response to different genotoxic stresses including alkylation, ultraviolet light, ionizing radiation, and replication inhibitors the 9-1-1 complex is loaded onto chromatin by a checkpoint clamp loader, composed by Rad17 protein and four small subunits of replication factor C (RF-C_{2-5}) (12-16). Besides its well documented role in DNA damage signaling, the 9-1-1 complex also directly participates in DNA metabolism. The checkpoint clamp physically associates and/or stimulates several components of base excision repair (BER) machinery (17-25) and additionally interacts with translesion Pols (26,27). The 9-1-1 complex functions have been also shown to be required for DSBR, HR and NER (28-31). These findings suggested a broad role of the 9-1-1 complex in multiple DNA transactions and a model in which the checkpoint clamp acts as recruiting platform for different factors involved in checkpoint response as well as DNA repair (32).

5.2.2. Structure

The 9-1-1 complex is a heterotrimeric, ring-shape protein complex of a predicted molecular weight 110 kDa. Based on genetic and biochemical data as well as molecular modeling techniques the model was proposed where Rad9 (45 kDa), Rad1 (31 kDa) and Hus1 (32 kDa) interact with each other in a head-to-tail manner like monomers within the PCNA ring (Figure 1) (9). The ring has a diameter of 10±2 nm with a 2–3-nm hole in the center. Several reports indicate that in cytoplasm Rad1 forms a heterodimer with Hus1 prior to its association with Rad9 (3,6,33). The remaining portion of hHus1 protein undergoes a rapid degradation via the Ub–proteasome pathway. The hRad1–hHus1 heterodimer subsequently translocates into the nucleus upon its association with hRad9 containing a C-terminal NLS. Through this process, a quality-controlled 9-1-1 checkpoint complex is formed and loaded onto chromatin by the hRad17–RF-C_{2-5} clamp loader when DNA damage is recognized (33). Importantly, the integrity and the function of the checkpoint clamp depends on the presence of all three subunits (34). It has been shown that in Hus1-null mouse embryonic fibroblast (MEF) cells the level of Rad9 was significantly reduced and detected only in cytoplasm indicating that the nuclear import and chromatin binding of Rad9 relied on Hus1 (34). Consistently, the nuclear localization of the fission yeast Hus1 requires Rad1 and Rad9 (4). Moreover, the damage-induced hyperphosphorylations of Rad9 as well as Rad17 were completely abolished in the absence of Hus1.

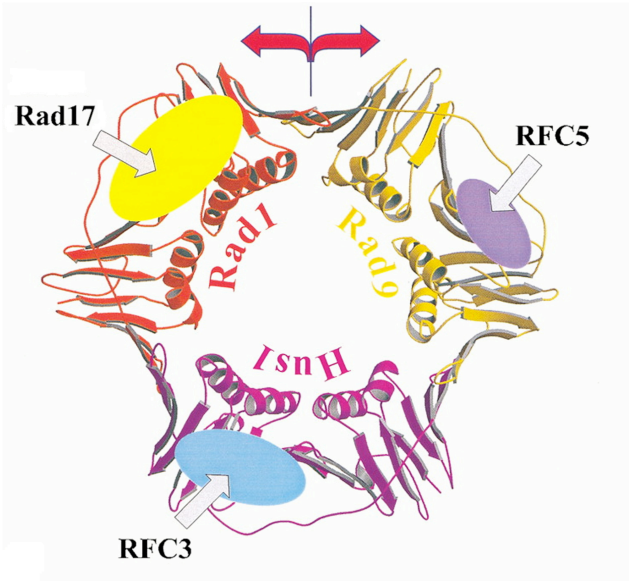

Figure 1. Structure of the Rad9-Rad1-Hus1 (9-1-1) clamp and topography of the interactions between subunits of the checkpoint clamp loader, Rad17-RF-C complex. The white arrows indicate the interaction sites with Rad17-RF-C_{2-5} subunits. From: Venclovas et al. Protein Sci 2002; 11: 2403-2416

5.2.3. The Rad9 protein

Human Rad9 was originally identified as a structural homologue of yeast *Schizosaccharomyces pombe* Rad9 which can partially complement radiosensitivity, hydroxyurea sensitivity, and associated checkpoint defects of *rad9* null yeast (35). The human *rad9* gene encodes a 391-amino acid long, 43 kDa protein that is 25% identical and 52% similar to the yeast protein (35). Rad9 is a nuclear protein, involved in multiple cellular processes (11,36). Biochemical studies have demonstrated that Rad9 possesses 3' → 5' exonuclease activity (37). In mammalian cells the protein mediates G_2-M and S-phase checkpoint activations by regulating the phosphorylation of Rad17, Chk1, Chk2 and Smc1 in response to replication block and DNA damage (38). Furthermore, Rad9 is essential for the maintenance of chromosomal stability and integrity. Rad9 knockout mice have been shown to be

embryonic lethal and cells with inactivated mammalian Rad9 had higher frequencies of chromosomal abnormalities, such as chromosomal end-to-end associations and chromosomal breaks (28,38). In mammalian cells, Rad9 is highly phosphorylated both constitutively and inducible after DNA damage (11,39). Additional phosphorylation events occur predominantly in the 110 amino acid carboxy-terminal (C-terminal) domain, which falls outside of the Rad1 and Hus1 interaction (40,41). Numerous phosphorylation sites (>10) have been mapped in this domain, and a subset of them are required for phosphorylation of Chk1 as well as interaction with TopBP1, which contains eight phosphopeptide binding BRCT domains.

5.2.4. The Rad1 protein

The human *rad1 gene* encodes a 283 amino acid long, 32-kDa protein that is predicted to be highly acidic and possesses 31% identity and 42.6% similarity to spRad1. Three Rad1 alternative splice variants with different open reading frames have been identified; one encodes a long form, hRad1A, and the two others encode shorter forms because of N-terminal truncation, hRad1B and hRad1C (42). Purified hRad1A has been show to exhibit 3' → 5' exonuclease activity, whereas other two variants lack this proof-reading activity. *In vivo,* Rad1 is essential for sustained cell growth and loss of Rad1 causes destabilization of Rad9 and Hus1 and consequently disintegration of the sliding-clamp complex. In response to DNA damage Rad1 undergoes phosphorylation modification in ATR- and TopBP1-dependent manner (36,43). In Rad1-depleted cells, ATR-dependent Chk1 activation was impaired whereas ATM- mediated Chk2 activation was unaffected, suggesting that the sliding clamp is required primarily for ATR-dependent signal activation. Disruption of sliding-clamp function also caused a major defect in S-phase control. Rad1-depleted cells exhibited chromosomal abnormalities and radioresistant DNA synthesis (RDS) phenotype, indicating that damage-induced S-phase arrest was compromised by Rad1 loss. Furthermore, lack of Rad1 also affected the efficiency of replication recovery from DNA synthesis blockage, resulting in a prolonged S phase (44).

5.2.5. The Hus1 protein

The mammalian Hus1 protein is a 280 amino acid long, 31kDa protein and shares 30% amino acid identity with the *S. pombe* protein. It is expressed throughout embryonic development and displays an ubiquitous expression pattern in adult tissues. It has been shown that the protein undergoes ATR-dependent phosphorylation in Xenopus egg extracts and in *S. pombe* although it is not clear whether the human Hus1 is phosphorylated as well (43). Targeted disruption of the mouse *Hus1* gene results in embryonic lethality and Hus1 -/- MEFs display an abrogated checkpoint response to UV radiation and replication blocks as well as increased apoptosis. These phenotypes were associated with a significantly increased frequency of chromosomal abnormalities and an S-phase–specific accumulation of phosphorylated histone H2AX, an indicator of double-stranded DNA breaks (45,46). Hus1 was also found to be essential for fragile site stability, since spontaneous chromosomal abnormalities occurred preferentially at common fragile sites upon conditional Hus1 inactivation (47).

5.3. The checkpoint clamp loader, Rad17-RF-C_{2-5} complex,

5.3.1. General overview

The Rad17-RF-C_{2-5} complex is a checkpoint-specific structural homolog of the replication factor, RF-C. The replicative form of RF-C is a heteropentamer composed of p140, p40, p38, p37, and p36. In Rad17-RF-C_{2-5}, the p140 subunit is replaced by the 75-kDa Rad17 protein (7,48). Electron microscopy has revealed that the two complexes have similar structures: a globular shape with a deep groove running down the length of the complex (Figure 2) (5,8,9,49). Genes encoding each of the subunits are essential, and all five subunits are required for efficient loading of the respective clamp (the 9-1- complex and PCNA, respectively) onto DNA. All five RF-C subunits are distinct but they all display significant sequence similarity with each other as well as with Rad17 and Ctf18, a large protein required for high-fidelity

chromosome segregation (5,9,48-52). Both proteins can replace the large subunit within the RF-C complex. The highest homology between RF-C subunits is concentrated within a region containing seven conserved sequences motifs referred to as RF-C boxes II–VIII (53). This high homology region links all RF-C subunits, Rad17, and Ctf18 protein families into a highly abundant and functionally diverse group of ATPases referred to as the AAA+ class (ATPases associated with various cellular activities) (54). Protein assigned to this class share also other conserved sequence regions among them the nucleotide-binding motifs Walker A (a phosphate-binding P-loop) and Walker B (often referred to as the DEXX motif).

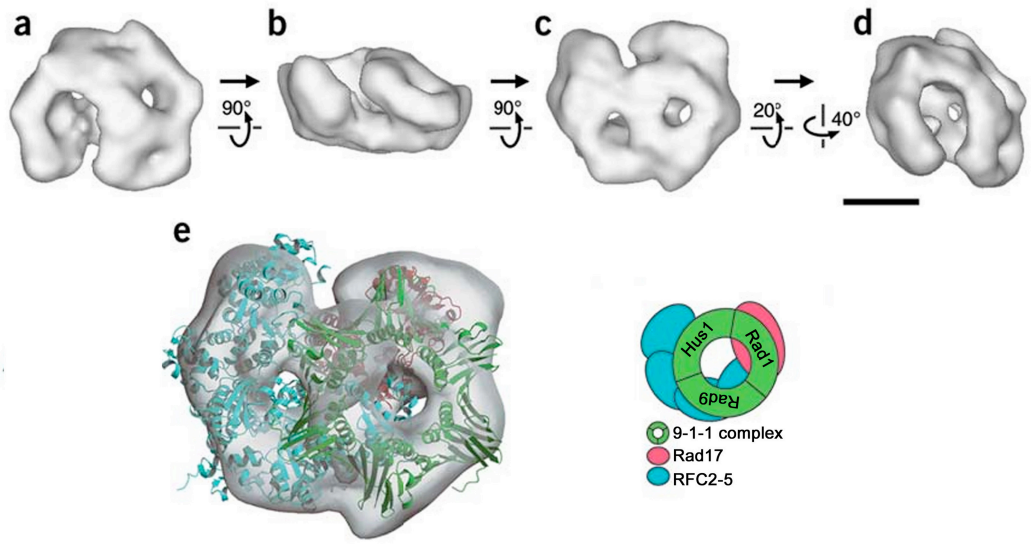

Figure 2. **Three-dimensional structure of the clamp-loading complex.**
(**a**−**d**) Various views of the surface representation of the three-dimensional reconstruction. (**a**) Top view. (**b**) Side view (**c**) Bottom view. (**d**) View at an oblique angle, related to **a** by a 20° rotation about the horizontal axis and a 40° rotation about the vertical axis. Scale bar, 50 Å. (**e**) Fitting of the crystal structures of the 9-1-1 complex and Rad17RF-C_{2-5} into the three-dimensional model of the clamp-loading complex. From: Miyata et al., *Nature Structural & Molecular Biology* **11**, 632 - 636 (2004)

5.3.2. The Rad17 protein

The human *hrad17* gene encodes a 590 amino acids long, 75-kDa protein although four alternatively spliced Rad17 RNAs have been identified and designated as to FM1, FM2, FM3 and FM4. The expression of each varies in different tissues and cell cycle phases. At least three of them encode polypeptides that are detected in human tissue as 71 kDa (main), 73kDa and 62 kDa band although the specific functions of each isoform remain unclear (55). Amino acid sequence alignment revealed that hRad17 has 28.3% and 52.5% similarity with the *S. pombe* Rad17 protein, and 21.8% identity and 45.8% similarity to the budding yeast cell cycle checkpoint protein, Rad 24. Rad17 protein is not only important for ATR-mediated checkpoint but is also essential for the cell viability (56,57). Loss of Rad17 function leads to checkpoint signaling defect, chromosomal aberration and endo-reduplication at high rate (57). Moreover, based on the studies employing mouse models it has been proposed that Rad17 possesses an important role in cell growth ad/or differentiation as Rad17-deficient embryos died during early/midgestation (56). In human cells approximately 40% of hRad17 is constitutively chromatin-associated independent of cell cycle phase, start to DNA replication or treatment with genotoxic agents (58). In addition, it has been reported that the mutant lacking ATP-binding motif poorly associates with chromatin and is enable to efficient promote loading of the 9-1-1 complex onto chromatin (34). In response to DNA damage the protein is phosphorylated on serines 635 and 645 in an ATR-dependent manner and overexpression of the non-phosphorylable mutant of Rad17 has been shown to interfere with DNA-damage-induced cell-cycle arrest (59,60). Consistent with this observation, the Rad17 mutant that binds poorly to chromatin shows significantly less DNA-damage-induced phosphorylation (34). Interestingly, even though phosphorylation of Rad17 is required for DNA damage-induced checkpoint activation, the same residues are also phosphorylated in unperturbed S phase of proliferating cells (58). It has been shown that phosphorylated hRad17 is localized to the sites of ongoing DNA replication which indicates that hRad17 has a key role in the regulation of DNA replication and suggest that chromatin-associated, phosphorylated hRad17 may serve as a sensor of DNA replication progression (57,58). In addition it has been

reported that the phosphorylated form of hRad17 associates with Pol ε, a DNA replication enzyme that has also been implicated in cell cycle checkpoints (61,62) Although it is not known whether hRad17 phosphorylation regulates its interaction with Pol ε, this interaction provides a physical link between checkpoint and DNA replication proteins. Moreover, there is a conserved and direct interaction between TopBP1 in humans (Dpb11 in budding yeast), a subunit of Pol ε and hRad9 (Ddc1 in budding yeast) that also participate in activation of DNA damage response pathway (63-65). Thus, Pol ε interacts with both the checkpoint clamp-loader and the checkpoint clamp complex, providing a possible molecular mechanism for the proposed checkpoint activities of Pol ε and the participation of hRad17 in the regulation of DNA replication by monitoring replication fork stability and sensing replication blocks during unperturbed replication (58). This is further supported by the fact that the Rad17 and PCNA co-localize at the sites of DNA replication and the interaction between two proteins is enhanced by the hydroxyurea (HU) treatment. Another protein found to interact with Rad17-RF-C_{2-5} complex is replication protein A (RP-A) (16,66,67). Very recently it has been shown that the Rad17-complex can interact with Lig I, an important enzyme which ligation activity is required for DNA replication to join Okazaki fragments as well to complete the BER pathway (68). In contrast to the replicative clamp loader, the RF-C complex that inhibits DNA end joining, the Rad17- RF-C_{2-5} complex stimulated ligation activity of Lig I. It has been proposed that the interaction between RF-C and Lig I my be involved in the unloading of PCNA after the joining of adjacent Okazaki fragments (69,70). Similarly, the Rad17-RF-C_{2-5} complex may play a similar role in the unloading the 9-1-1 complex from DNA, thus switching off the checkpoint (68).

5.4. Topological model of the clamp loading process

In vitro and *in vivo* studies have shown that the Rad17-RF-C_{2-5} complex is required for efficient loading of the checkpoint clamp, the 9-1-1 complex onto DNA (12,14,34,71,72). Based on the biochemical data as well as computationally derived molecular models the topological model of clamp loading process has been proposed (Figure 3) (49). Upon ATP binding, the Rad17-RF-C_{2-5} complex undergoes an initial conformational change exposing the clamp-interacting regions of the individual subunits (73). Subsequently, Rad17 binds to the C-terminal region of the Rad1 protein, destabilizing the adjacent interface of the clamp. Binding of the other subunits, particularly of RF-C3 and possibly RF-C5, to the C-terminal regions of the Hus1 and Rad9 monomers, respectively, temporarily fixes the clamp in the state with the open interface positioned between Rad17 and RF-C5. This ATP-bound quasi-stable structure of the complex between the clamp loader and the clamp has a high affinity to the double-stranded/single-stranded DNA structure. When DNA binds, its single-stranded portion triggers ATP hydrolysis with subsequent ADP dissociation. That resets the conformation of the clamp loader, causing the release of the clamp, which then spontaneously closes, trapping DNA inside (Figure 3).

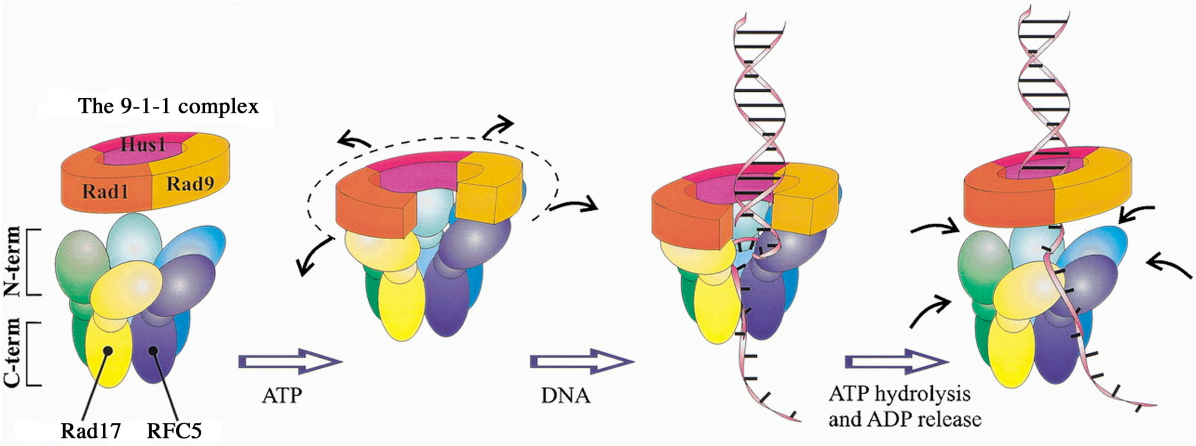

Figure 3. Schematic view of the RF-C- or Rad 17-RF-C_{2-5}-dependent loading of PCNA or the 9-1-1 complex onto DNA. For details see text. From: Venclovas et al. Protein Sci 2002; 11: 2403-2416

5.5. The role of the Rad17-RF-C_{2-5} and 9-1-1 complexes in the checkpoint activation

In response to genotoxic stress, cells initiate cell cycle checkpoints that regulate cell cycle progression, trigger apoptosis, and influence DNA repair (74). The initiation of these checkpoints relies on the efficient detection of DNA lesions followed by rapid signal transduction cascade. In response to specific forms of genotoxic stress, unique sensor proteins detect damage and trigger cell cycle arrest. The ataxia telangiectasia mutant (ATM) and the ATM and Rad3-related (ATR) belong to the group of phosphoinositide 3-kinase-related kinases (PIKKs) and their functions are necessary for maintaining fidelity of these checkpoint pathways (75-78) . Following exposure to ionizing radiation (IR) or DNA double strand breaks, ATM becomes rapidly activated and phosphorylates a number of protein involved in checkpoint initiation including Chk2, p53, NBS1, BRCA1 and itself (1,75,77). In contrast, ATR responds to a broad spectrum of DNA damage caused by ultraviolet (UV) light and replication disruption (79-81). The DNA damage-induced phosphorylation of Chk1, the downstream effector kinase of ATR, requires the ATR-interacting protein (ATRIP), Claspin, and the Rad17 and the 9-1-1 complexes (82). Loss of Rad17 or Rad1 abrogates ATR-dependent, but not ATM-dependent, signaling. Activated Chk1 plays critical roles in cellular checkpoint responses by stabilizing stalled replication forks, blocking the firing of late origins of replication, and arresting cells in G2/M. This pathway is initiated when the replicative Pols stall and large tracts of single-stranded DNA are created by the uncoupling of the replicative MCM helicase complex from the advancing replication fork (83). The single-stranded DNA is then coated by RP-A which signals the independent recruitment of ATR-ATRIP, Rad17-RF-C_{2-5}, the 9-1-1 complex, Cut5 and claspin. The ATRIP-ATR complex is bound to DNA by a direct interaction between ATRIP and RPA, and the binding is additionally stimulated by Cut5 (84). Cut5 has been also shown to facilitate the recruitment of Pol α, which in turn recruits Rad17-RF-C_{2-5} (83,85). It has been also proposed that chromatin associated Rad17- RF-C_{2-5} complex interacts with the MCM complex in the vicinity of the replication fork and this interaction is required for the replication checkpoint signaling (86). RP-A also interacts with Rad17- RF-C_{2-5} complex and stimulates the

binding of the clamp loader as well as the loading of the 9-1-1 complex. Rad17- RF-C_{2-5} loads the 9-1-1 complex onto chromatin in a reaction that is analogous to the loading of PCNA onto sites of DNA replication (12,14). Chromatin associated Rad17 is phosphorylated by ATR on serines 635 and 645 (34,59). Importantly, ATR is primarily, if not entirely, responsible for the UV-induced phosphorylation of Rad17 but HU and IR could induce phosphorylation of Rad17 even in the absence of ATR, suggesting that ATM might also phosphorylate Rad17 *in vivo.* Moreover, phosphorylated Rad17 interacts and stimulates Claspin, a key regulator of Chk1. Although the binding of the ATRIP–ATR complex and the loading of the 9-1-1 complex occur independently, both events are essential for optimal ATR-mediated Chk1 phosphorylation and activation (87). Recently it has been proposed that the role of the 9-1-1 complex in Chk1 activation is to localize TopBP1, bound to the phosphorylated Rad9 tail to the stalled fork. The Rad9-tethered TopBP1 can then interact with the ATRIP/ATR and stimulate ATR-mediated Chk1 phosphorylation, and cell survival (63). Importantly HU and IR could induce phosphorylation of Rad17 even in the absence of ATR, suggesting that ATM might also phosphorylate Rad17 *in vivo* (Figure 4).

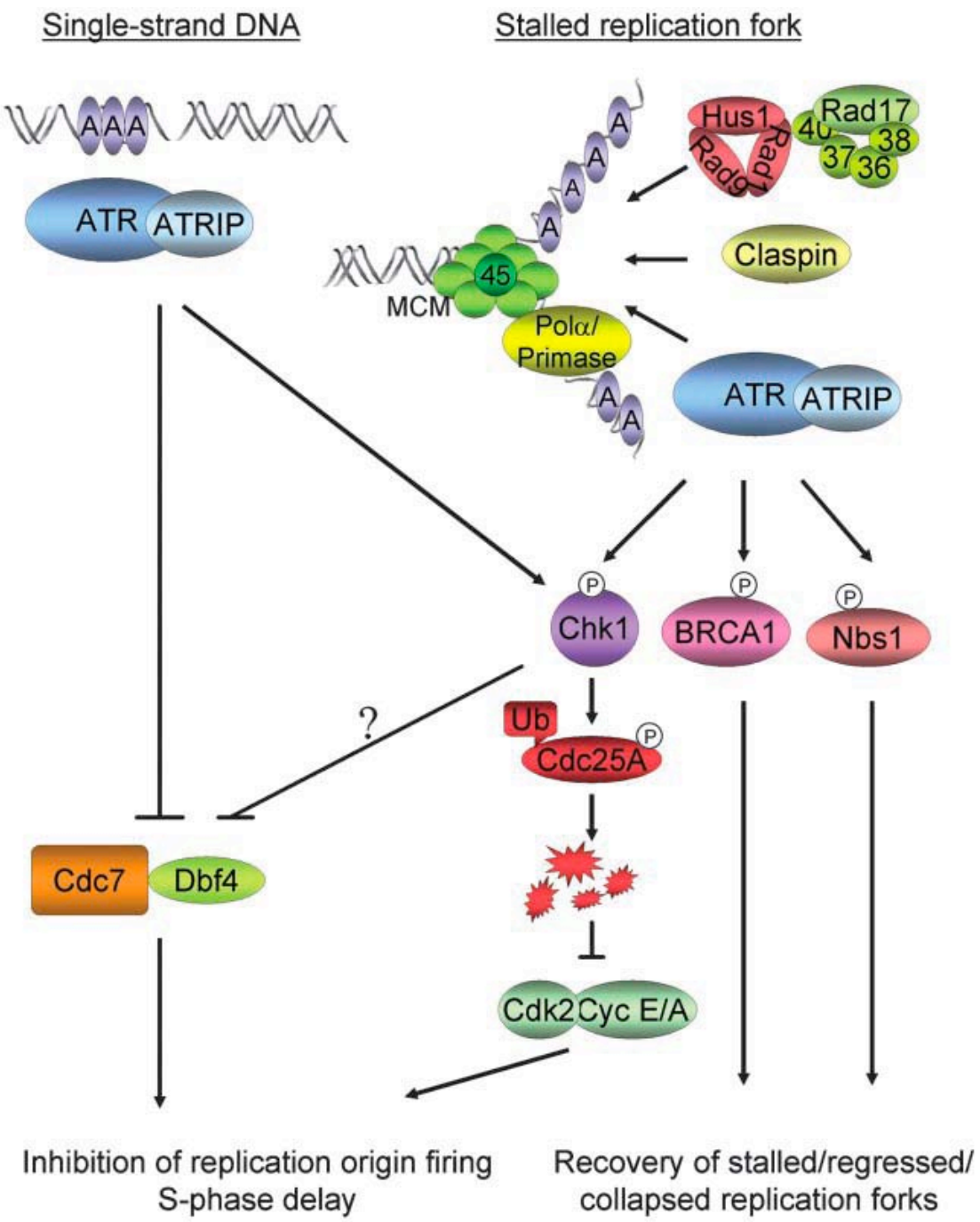

Figure 4. The ATR-mediated intra-S check-point. For details see text.
From: Sancar et al., Annu Rev Biochem. 2004;73:39-85

5.6. The Base Excision Repair (BER) Pathway

BER is one of the most active DNA repair processes. This pathway is responsible for processing small base lesions formed in DNA via spontaneous hydrolysis, attack of reactive oxygen species (ROS), or modification by alkylating agents (88). It has been estimated that in a mammalian cell, 10^4 damaging events occurs every day, which, emphasizes the importance of the BER pathway. Since ROS are products of normal cellular metabolism, they generate the majority of damages processed by the BER pathway, including not only base modifications, but also sites of base loss and DNA strand breaks. As many of the BER substrates are mutagenic, this repair pathway plays an essential role in maintaining genomic integrity. BER consists of five main steps catalyzed by different enzymes. The first step is initiated by a lesion-specific DNA glycosylase (mono- or bifunctional) and followed by either of two sub-pathways: short-patch (SP) BER; a mechanism where only 1 nucleotide is replaced or long-patch (LP) BER; a mechanism resulting in the replacement of 2–13 nucleotides (89). The majority of repair is currently thought to occur via the SP-BER pathway initiated by either a mono-functional or bi-functional glycosylase. In the simplified model for the SP-BER pathway, a mono-functional glycosylase excises base lesion, leading to the formation of an AP site, subsequently hydrolysed by APE1 (90). APE1 catalyzes the incision of the damaged strand via Mg^{2+} stimulated mechanism, leaving a 3'OH and a 5'deoxyribose-phosphate moiety (5'dRP) at the margins. Pol β hydrolyzes the 5-dRP moiety and fills the single nucleotide gap, preparing the strand for ligation by either Lig I or a complex of Lig III and XRCC1. However, oxidative base lesions are primarily removed by bi-functional DNA glycosylases that also possess an additional 3'AP lyase activity (89,90) . Upon recognition of a damaged base by a bi-functional DNA glycosylase, the lesion is excised from the DNA strand by a mechanism similar to the one utilized by monofunctional DNA glycosylases. However, the DNA backbone can then be incised 3' to the damage site, resulting in a 3' α,β-unsaturated aldehyde (after β -elimination) and a 5' phosphate at the termini. A 3'phosphodiesterase activity of APE1, cleaves this terminus, followed by DNA synthesis by Pol β and ligation by LigI or

XRCC1/LigIII (91). Long-patch BER is initiated in a manner similar to SP-BER to produce a nicked DNA intermediate. Repair completion requires a 3'OH moiety for proper nucleotidyl transfer and chain elongation. In cases where the 5'lesion is refractory to Pol β lyase activity the next step is carried out by Pol δ, ε, or β coupled with PCNA and a variety of other proteins including Fen 1, poly (ADP-ribose) polymerase 1 (PARP1) and Lig I synthesizes DNA to fill the gap, resulting in a displaced DNA flap of 2–13 bases in length (92,93). DNA synthesis and strand displacement by Pol β is stimulated by the combined presence of Fen 1 and PARP1 (94). Fen 1 then catalyzes the removal of the ensuing DNA flap, leaving a nick that has been transferred 2–13 nucleotides downstream of the original damage site. Finally, the intact DNA strand is restored by Lig I (Figure 5).

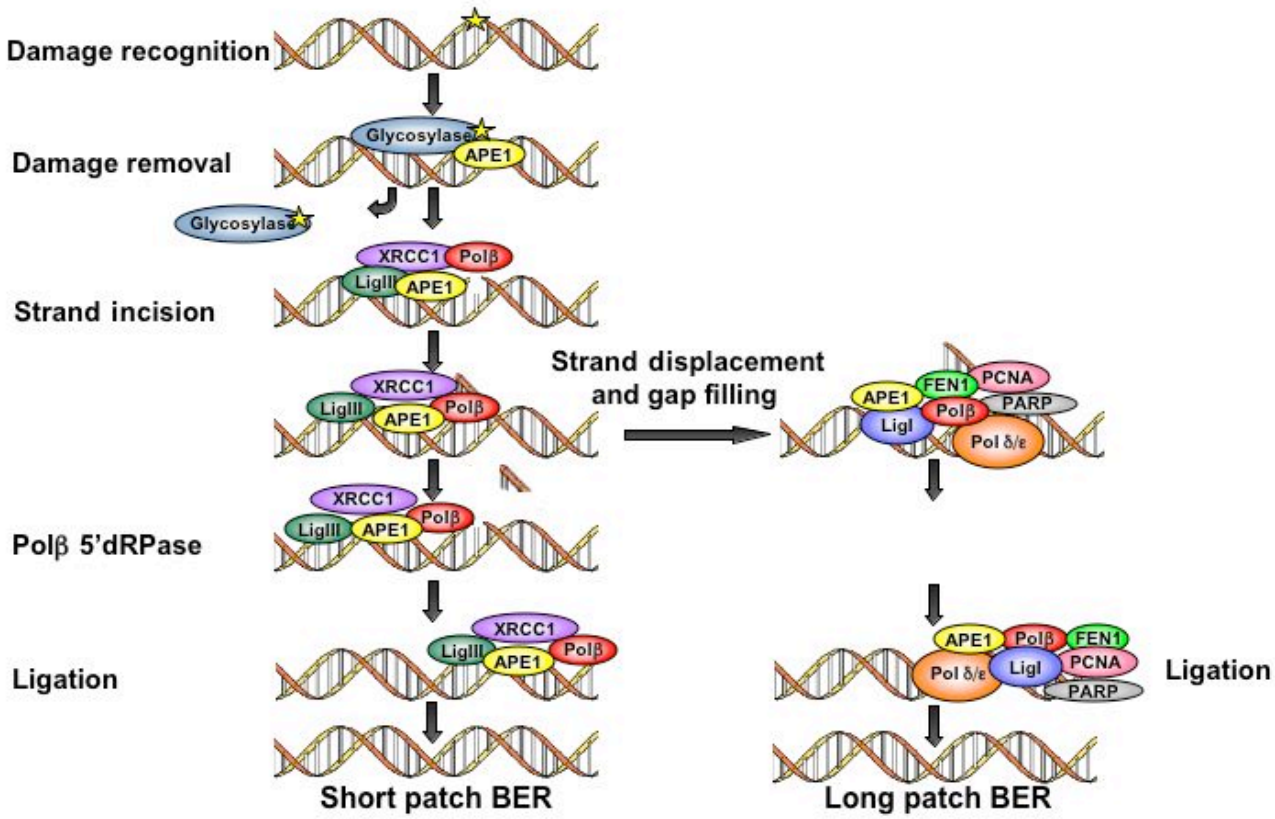

Figure 5. Classical model of the SP- and LP-BER sub-pathways.

For details see text.

5.6.1 Apurinic/apyrimidinic endonuclease 1 (APE1)

AP endonucleases are classified into two families according to their homology to *E. coli* endonucleases: exonuclease III (*x*th) and endonuclease IV (nfo) (95). The first includes exonuclease III (*E. coli*), Exo A (*Streptococcus pneumoniae*), Rrp 1 (*Drosophila melanogaster*), Arp (*Arabidopsis thaliana*), Apn2 (*S. cerevisiae*), APEX (mouse), BAP1 (bovine), rAPE (rat), chAPE1 (hamster), and APE1/Ref-1 (humans; previously referred to as HAP1 and APEX1). These enzymes exhibit strong AP hydrolytic activity and 3'-diesterase activity with APE1/Ref-1 having the highest 5'-endonuclease rate, but lowest 3'-diesterase activity. Typically, the exonuclease III family of endonucleases accounts for approximately 95% of the repair activity in the organism. The main members of the second family of endonucleases, the endonuclease IV family are Apn1 (S*. cerevisiae*), endonuclease IV (*E. coli*), Spapn1 (*S. pombe*), and CeApn1 (*C. elegans*). Human APE1/Ref-1 is 318 amino acids long and approximately 36kDa protein, sharing 25–40% sequence identity with prokaryotic homologues (95-97). APE1 contains two distinct domains: the N-terminal domain, which is essential for redox activity and contains the nuclear localization sequence and the C-terminal region responsible for the endonuclease activity in repair pathways resides (98-100) APE1 plays multiple roles in DNA metabolism. In addition to its role in BER, it is involved in oxidative DNA damage repair and stimulates the DNA binding activity of AP-1 (Fos, Jun) proteins as well as nuclear factor-κB (NF-κB), the polyoma virus enhancer-binding protein 2 (PEBP2), the early growth response-1 (Egr-1), Myb, members of the ATF/CREB family, the hypoxia inducible factor-1α (HIF-1α), the HIF-like factor (HLF), Pax-5 and Pax-8 (95). APE1 possesses also the major AP-1 redox activity in cells and represents a novel redox component of signal transduction cascades that regulates eukaryotic gene expression. APE1 has been also implicated in the control of p53 activity through redox dependent and independent mechanisms. Additionally, it has been also closely linked to apoptosis and altered levels of APE1 have been found in some cancers. In mammals, APE1 functions are essential, and APE1 -/- mice embryos die at the early stage of development (100-102). The multifunctional properties of APE1 strongly correlate

with the differential expression pattern in different types of cells and tissue (96,103-105). At least five different patterns of APE1/Ref-1 staining have been reported. First, regional variation in the expression level of APE1/Ref-1 is a commonly observed feature. Second, APE1/Ref-1 is found in the nucleus of cells where it is probably, involved in DNA repair. The third staining pattern is cytoplasmic and it has been found in macrophages, spermatocytes, hippocampal cells, hepatocytes, hypoglossal motor neurons, and breast cells (105-108). Cytoplasmic presence of APE1 may reflect its role as a DNA repair protein in the mitochondria or alternatively as a redox protein, important to maintain newly synthesized transcription factors in a reduced state while they are being transported to the nucleus (105,109). Interestingly, in the cytoplasm of hypoglossal motor neurons, the APE1/Ref-1 staining is associated with ribosomes suggesting a role in ribosomal function (106) . Moreover, APE1 is found in cytoplasm of proliferating or highly metabolitically active cells that often experienced an increase in oxidative stress and the presence of APE1/Ref-1 in the cytoplasm may reflect an involvement in cellular responses to oxygen stress. The fourth pattern of staining shows differential localization within cell subpopulations, further demonstrating the complexity of APE1/Ref-1 expression. For example, in many cerebellar granule cells, APE1/Ref-1 staining is only cytoplasmic, while adjacent cells exhibit intense nuclear staining (105). Finally, many cell types exhibit both cytoplasmic and nuclear localization of APE1/Ref-1, including adrenal cortical cells, cerebellar purkinje cells, some cervical cells, pneumocyes, and parietal and mucosal cells of the stomach (105,107). APE1 has been also reported to undergo posttranslational modifications including acetylation, phosphorylation, and oxidation. Acetylation of APE1 by p300 stimulates its co-repressor activity but had no effect on the endonuclease activity of the protein (110). APE1 has been also shown to be phosphorylated *in vitro* by PKC, CKI and II although the role of phosphorylation in regulating the activities of APE1 remains unclear (111,112). On the other hand, oxidation/reduction status of APE1 influences its activity since AP endonuclease activity of purified APE1 was dramatically reduced in the presence of the oxidizing agent hydrogen peroxide or diamide, with minimal protein degradation (113). Overall, APE 1 appears to form a link between the BER pathways, cancer, and regulation of

transcription factors, oxidative signaling and cell cycle control. Figure 6 shows the structure of APE1 bound to DNA.

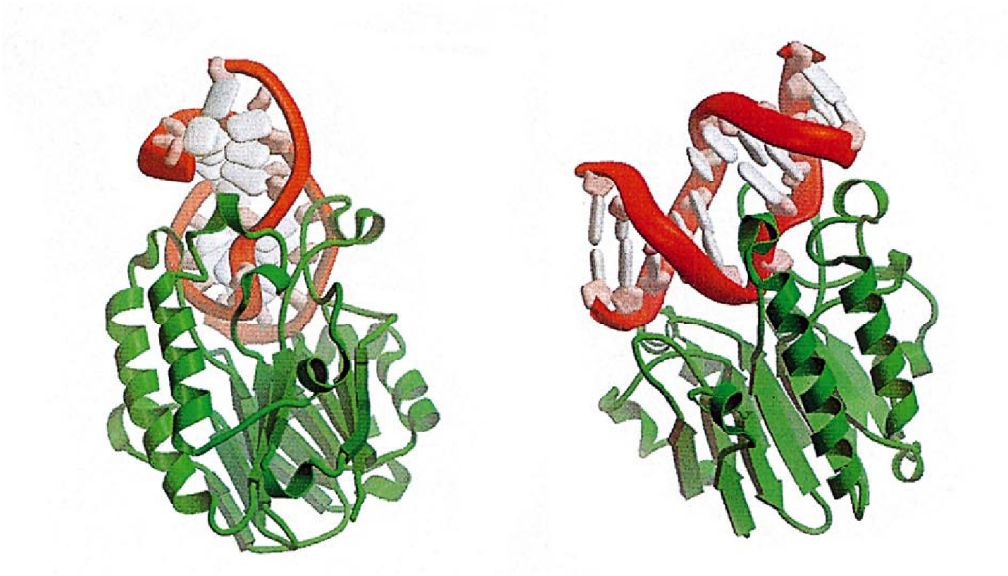

Figure 6. **Crystal structure of APE1 bound to DNA.** From: Wilson et al., Nat Struct Biol. 2000 Mar;7(3):176-8

5.7. References

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5.8. The checkpoint sensors, the 9-1-1 complex and the Rad17-RF-C_{2-5} complex: At the crossroad of DNA damage checkpoints and DNA repair (Review)

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The checkpoint sensors, the 9-1-1 complex and the Rad17-RF-C_{2-5} complex: At the crossroad of DNA damage checkpoints and DNA repair

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Abstract

Upon exposure to genotoxic stress cells activate checkpoint-signaling pathways that halt cell cycle progression, regulate DNA repair and trigger apoptosis. Studies in yeast and humans indicate that Rad17, Rad9, Rad1, Hus1 play key roles in checkpoint activation. Three of these proteins Rad9, Hus1, and Rad1 form a heterotrimeric complex (the 9-1-1 complex), which resembles a PCNA-like sliding clamp, whereas Rad17 is part of a clamp-loading complex that is related to the PCNA clamp loader, replication factor-C (RF-C). In response to DNA damage, the 9-1-1 complex is loaded onto DNA in Rad17-RF-C_{2-5}-dependent manner. The DNA-bound 9-1-1 complex then facilitates ATR-mediated phosphorylation and activation of Chk1, a protein kinase that regulates S-phase progression, G2/M arrest, and replication fork stabilization. In addition to its role in checkpoint activation, a growing number of evidence suggests that the 9-1-1 complex also directly participates in DNA repair by interacting and/or stimulating several components of different repair pathways including base excision repair (BER), nucleotide excision repair (NER), double strand breaks (DSBs) repair and translesion synthesis (TLS). In this review the involvement of the checkpoint clamp, 9-1-1 complex and the checkpoint clamp loader, Rad17-RF-C_{2-5} in different repair pathways is discussed.

Introduction

The integrity of the eukaryotic genome is maintained by coordinated actions of cellular pathways including DNA repair, DNA damage and replication checkpoint, chromatin remodeling and apoptosis. It has been estimated that approximately 10,000-100,000 modifications occur daily in the DNA of a single cell (1). The major repair pathway protecting cells against a single-base DNA damage caused by attack of reactive oxygen species (ROS), spontaneous hydrolysis, or modification by alkylating agents is base excision repair (BER) (2), although other pathways such as nucleotide excision repair and methyltransferase-mediated error-free repair are also involved (3).

The base excision repair (BER) pathway

BER is often initiated by a damage type specific DNA glycosylase that cleaves the *N*-glycosydic bond of the damaged base, leaving an apurinic/apyrimidinic site, also referred to as an abasic site or AP site (4). Subsequently, AP endonuclease 1 (APE 1) cleaves the sugar-phosphate backbone at the 5'-side of the AP site resulting in 3'-hydroxyl and 5'-deoxyribose phosphate (dRP) groups flanking a one-nucleotide gap (5). DNA polymerase β (Pol β) inserts a first nucleotide into the gap, leaving nicked DNA with a 5'-dRP flap (6). At this point, the repair can be accomplished via two different BER sub-pathways depending on the nature of the AP site (2). In case of regular AP sites, Pol β removes the 5'-dRP group through its associated dRP-lyase activity (7), and the resulting nick is sealed by the DNA ligase III/XRCC1 complex. This sub-pathway is referred to as short-patch BER (SP-BER). However, if the sugar group is oxidized or reduced, Pol β cannot remove the 5'-dRP moiety and repair proceeds through the alternate long-patch BER (LP-BER) sub-pathway involving removal and replacement of 2-10 nucleotides (3,8). It has been found that, in the LP-BER pathway, the collaboration of Pol β /, proliferating cell nuclear antigen (PCNA), replication factor C (RF-C) and flap endonuclease 1 (Fen 1) can displace the strand 3' to the nick and synthesize up to 10 nucleotides. The resulting flap is cut by Fen 1 and the final nick is sealed by DNA ligase I (Lig I) ((9-13). However, growing evidence indicates that Pol β is a key enzyme in BER, that not only initiates both sub-pathways (14) but can also perform efficient strand displacement via the "hit and run"

mechanism (4). In this model Pol β and Fen 1 act successively, followed by the action of Lig I.

DNA damage checkpoints

DNA repair is coordinated with cell-cycle progression (1,15) and with DNA-damage checkpoints (16). DNA-damage checkpoint pathways are activated at specific points during the cell cycle when the integrity of DNA is examined before progression to the next cell-cycle phase is allowed. The mechanism of checkpoint response is based on a signal transduction cascade, where sensor proteins detect a lesion in the double helix and stimulate several effectors through the activity of different protein kinases. This may lead to temporary cell cycle arrest, slowing down of DNA replication, changes in the cellular transcriptional program, chromatin remodeling, induction of DNA repair genes and occasionally in apoptosis (17,18). Some of the major checkpoint proteins namely ataxia telangiectasia mutated protein (ATM), ATM-related protein (ATR), ATR interacting protein (ATRIP), Rad17, Rad9, Rad1 and Hus1, are thought to be involved in sensing and triggering DNA repair processes. Among those, the three human proteins Rad9, Hus1, and Rad1 form a heterotrimeric complex (the 9-1-1 complex) which exhibits structural similarity with the homotrimeric clamp formed by PCNA (19-21). The 9-1-1 complex has been characterized as a sensor of DNA damage and is targeted to damage sites following genotoxic stress (17,22-27). In addition, Rad17 associates with the four small subunits of the heteropentameric RF-C complex in a manner similar to the RF-C complex (20,21,28-30). Several studies showed that the 9-1-1 complex and Rad17-RF-C_{2-5} function as a clamp/clamp–loader pair, similarly to PCNA and RF-C_{2-5} (22,23,27,31,32). Based on the *in vitro* and *in vivo studies* it has been proposed that the Rad17-RF-$C_{2–5}$ complex recognizes DNA lesions, allowing the recruitment of the 9-1-1 complex to those sites. ATM and ATR kinases are recruited simultaneously to the same sites of DNA damage but in a 9-1-1 complex and Rad17-RF-$C_{2–5}$ independent manner (33,34). The current model suggests that the 9-1-1 complex may facilitate the recruitment of the checkpoint effector kinase Chk1 (24,26) that is subsequently phosphorylated by the ATR/ATM kinases (17). Additionally, it has been recently proposed by two different groups, that the 9-1-1 complex and the Rad17–RF-C_{2-5} alternative clamp

loader could stabilize stalled replication forks (23,27). Despite to their well-documented roles in checkpoint sensing and signaling, several reports have implicated the 9-1-1 complex and Rad17-RF-$C_{2–5}$ in various DNA repair pathways. Below, recent findings are presented indicating possible connections between the checkpoint clamp/clamp loader pair and several components of various DNA repair pathways thus providing direct links between DNA damage response and DNA repair.

Physical and functional interaction between two checkpoint sensors, the 9-1-1 complex and the Rad17-RF-C_{2-5} complex and BER components.

Recent investigations indicate that two essential checkpoint sensors, the 9-1-1 complex and Rad17-RF-C_{2-5} complex directly participates in different repair pathway. The 9-1-1 complex has been implicated in translesion synthesis which utilizes the "error-prone" polymerases that are recruited to the sites of lesion where Pol d is unable to synthesize DNA (35-38). It has been proposed that, the 9-1-1 complex besides its function in checkpoint signaling through the ATM and ATR kinases could also act as a recruiting platform for translesion polymerases at the stalled replication forks (39). In support to this hypothesis, *S. pombe* Rad17 was shown to be required for binding of the translesion synthesis (TLS) Pol DinB to chromatin where DinB physically interacts with the 9-1-1 complex. Pol z is the second TLS Pol known to interact *in vivo* and *in vitro* with the 9-1-1 clamp (38). It has been suggested, that the 9-1-1 complex may physically regulate Pol z dependent mutagenesis by controlling the access of Pol z to damage DNA. Moreover, the 9-1-1 ways also implying the translesion synthesis past UV-induced DNA damage (37). A role of the 9-1-1 complex in response to DNA DSBs has also been demonstrated. Deletion of the 9-1-1 complex genes lead to an increased sensitivity to enzymatically produced DSBs in *S. cerevisiae*. Moreover the DNA damage-induced co-localization of 9-1-1 complex and Rad22 in fission yeast has been described (40). Rad22, the major homologue of Rad52 protein family in *S.pombe*, is involved in homologous recombination and in other mechanisms of DSBs repair, such as single strand annealing. Furthermore, the Rad9 subunit of the checkpoint clamp was implicated in the homologous recombinantional repair as it interacted with Rad51, a protein catalyzing strand

invasion during homologous repair (41). Direct interactions between ScRad14 (human xeroderma pigmentosum group A, hXPA), a DNA binding protein, involved in NER and the checkpoint proteins ScDdc1 (the hRad9 homologue) and ScMec3 (the hHus1 homologue) have been demonstrated (42).

Recent studies have demonstrated that the 9-1-1 complex can interact and/or stimulate most components of BER machinery. The DNA glycosylase MutY (MYH) is involved in oxidative DNA damage repair. The enzyme excises adenine bases from the DNA backbone at sites where adenine is inappropriately paired with guanine, cytosine, or most importantly 8-oxo-7,8-dihydroguanine, a major DNA lesion after oxidation damage. Recently it has been shown that MYH directly interacts with the 9-1-1 complex in both the fission yeast *Schizosaccharomyces pombe* and human cells (43,44). The association between the 9-1-1 clamp and MYH occurs predominately via its Hus1 and Rad1 subunits as shown by pulldown assays. The physical interaction between two proteins was also confirmed *in vivo* by co-immunoprecipitation and co-localization studies. Furthermore this interaction was enhanced upon genotoxic stress conditions and correlated with genotoxin-induced phosphorylation of Hus1. In addition, the 9-1-1 complex enhanced the glycosylase activity of MYH. Notably, physical and functional interactions of MYH–Hus1 and MYH–Rad1 from *S. pombe* and human cells are interchangeable, similarly to the previously reported MYH-PCNA interaction (45-47). NEIL1 is the second DNA glycosylase that physically and functionally interacts with the 9-1-1 complex (48). NEIL1 is also involved in repairing of oxidatively damaged DNA bases and its main target are oxidized pyrimidines. The 9-1-1 complex interacted with NEIL1 *in vivo* and *in vitro* via all three monomers of the complex and the C-terminal domain of NEIL1. Moreover, a significant fraction of the hNEIL1 nuclear foci co-localized with hRad9 foci in hydrogen peroxide treated cells. Human NEIL1 DNA glycosylase activity was also significantly stimulated by the 9-1-1 complex (48). Another component of BER pathway which activity was shown to be influenced by the 9-1-1 clamp is APE1 (49). The 9-1-1 complex was shown to interact with APE1 *in vitro* and specifically stimulate the endonuclease activity of the protein on a single enzyme assay as well as in the reconstituted LP-BER *in vitro*, independently of its loading onto DNA. Co-

immunoprecipitation experiments using human, total cell extract further confirmed that the two proteins interact *in vivo.* Moreover upon exposure to genotoxic stress, the 9-1-1 complex and APE1 co-localized at the sites of DNA damage where the 9-1-1 complex was proposed to further stimulate endonuclease activity of APE1 in BER. Interestingly. APE1 was also reported to interact with the checkpoint clamp loader, the Rad17-RF-C_{2-5} complex (Gembka et al, manuscript in preparation). Previously it has been shown that *in vitro,* the loading of the 9-1-1 complex is not absolutely required for the stimulation of APE1 activity and the observed stimulation could result from protein-protein that does not involve the encircling of the DNA substrate (49). However, one should bear in mind that proposed model might still differ from the real *in vivo* situation especially only the loading of the 9-1-1 complex onto chromatin by the Rad17-RF-C_{2-5} complex leads to the local increase of its concentration at the damage sites, where it further functions in the DNA damage signaling and most probably, in DNA repair. Indeed, APE1 was shown to interact with Rad17-RF-C_{2-5} complex in *vivo* and *in vitro* as revealed by immunoprecipitation experiments using human, total cell extracts and pulldown assays with purified proteins. Moreover, APE1 and Rad17 co-localized to the same nuclear foci in human cells and this interaction was enhanced upon DNA damage. Furthermore, physical and functional interactions between the 9-1-1 complex and Pol β have been also reported. *In vitro* experiments using purified proteins revealed that the 9-1-1 clamp exerts a specific stimulatory effect on Pol β activity (50). The checkpoint clamp stimulated Pol β activity on primer-template by increasing its affinity for the 3'-OH primer end. The observed stimulatory effect was specific and limited to the Pol β activity only, since the replicative Pols α and δ were not influenced by the 9-1-1 complex. In addition, the 9-1-1 complex increased deoxyribonucleotides misincorporation by Pol β and stimulated DNA strand displacement synthesis on a 1 nucleotide template and in the complete LP-BER reconstituted *in vitro*, raising the possibility that the 9-1-1 complex might attract Pol β to DNA-damage sites. Interestingly, all three subunits of the 9-1-1 complex were able to interact with Pol β as shown by co-immunoprecipitation experiments and pulldown assays. Another BER component whose enzymatic activity is affected by the 9-1-1 complex is Fen 1 (51,52). It has been reported that the 9-1-1 clamp stimulates Fen 1 cleavage on the substrates containing different

length flaps (52) When both ends of the substrate were blocked with biotin-streptavidin, thus preventing the 9-1-1 clamp from sliding along DNA, the observed effect was strongly reduced indicating that the 9-1-1 clamp must be loaded onto DNA in order to exert its stimulatory effect. Both proteins were able to interact *in vivo* and *in vitro* as demonstrated by immunoprecipitation and pulldown experiments, respectively (51,52). Interestingly, the 9-1-1 appeared not to interact with Fen 1 through its PCNA-binding motif, raising the possibility that the two clamps might interacts with Fen 1 simultaneously but via different mechanism (51). Indeed, the presence of both clamps did not interfere with the binding or with the stimulatory effect that either of them exerted on Fen 1 cleavage efficiency. However, acetylation of Fen 1 completely abolished the 9-1-1 complex stimulatory effect whereas it did not influence PCNA stimulation of Fen 1 cleavage leading to the conclusion that the effects both clamp exert on Fen 1 enzymatic activity could be regulated via its post-translational modification of Fen 1. These findings, together with the fact that PCNA and the 9-1-1 complex co-localize upon DNA damage, suggest that the two DNA clamps could act simultaneously in some steps of the BER process. Since recruitment of the 9-1-1 complex onto chromatin upon DNA damage has been shown to be an early event of checkpoint activation (34,53) one may propose that upon binding to the lesion, the 9-1-1 complex may recruit and stimulate the BER proteins where DNA repair should occur. At the latter step of repair the presence of PCNA together with the network of protein-protein interaction within BER component could allow an increased efficiency of the repair process. This hypothesis is further supported by the observation that in the complete reconstituted LP-BER *in vitro* the 9-1-1 complex exerts its stimulatory effect on the two early components of BER, namely APE1 and Pol β whereas it does not stimulate the enzymatic activates of Fen 1 and Lig I (49). Interestingly, the activity of Lig I was also stimulated by the 9-1-1 complex in a single enzyme assay and the presence of the PCNA did not prevent this effect (54). It has been suggested that the 9-1-1 complex stimulated Lig I activity by promoting its the binding to the nicked substrate. However, the loading of the checkpoint clamp does not seemed to be absolutely required to stimulate Lig I activity, indicating that the observed effect could derive from a protein-protein interaction that does not involve the encirclement of the DNA structure (55). In

addition, both proteins were shown to interact *in vitro* as well as and *in vivo* and this interaction was enhanced upon exposure to genotoxic stress (54-56). Very recently a novel interaction between Lig I and the checkpoint clamp loader, Rad17-RF-C_{2-5} complex has been described (56). The complex interacted with Lig I *in vivo* and this interaction occurred via N-terminal domain of its large subunit, Rad17. Interestingly, similar interaction between Lig I and replicative clamp loader, RF-C have been observed (57). This interaction was also mediated via the large p140 subunit of RF-C, although the two complexes were shown to bind to different regions on Lig I. Moreover, RF-C exerts an inhibitory effect on Lig I activity whereas Rad17-RF-C_{2-5} stimulates the joining process. Similar results were obtained with the homologous *Saccharomyces cerevisiae* proteins indicating that the interaction between the replicative DNA ligase and checkpoint clamp is conserved in eukaryotes. Notably, the Rad17-RF-C_{2-5} complex was shown to preferentially interact and stimulate dephosphorylated Lig I. Moreover, Lig I and Rad 17 associated *in vivo* as demonstrated by immunoprecipitation studies and this association was enhanced in S phase following DNA damage and replication blockage that occurs concomitantly with DNA damage-induced dephosphorylation of chromatin-associated Lig I. Thus, it has been proposed that the *in vivo* interaction between Lig I and the checkpoint clamp loader is regulated by post-translational modification of Lig I (56). Importantly, once the damage is removed, it is important to switch off the checkpoint and restart replication. It seems possible that the interaction between RF-C and Lig I may be involved in the unloading of PCNA after the joining of adjacent Okazaki fragments (57,58) while Lig I and Rad17-RF-C_{2-5} may play a similar role in unloading the Rad9-Rad1-Hus1 complex, thereby switching off the checkpoint.

Conclusions

A growing number of evidence indicates that the checkpoint clamp, the 9-1-1 complex together with its clamp loader, Rad17-RF-C_{2-5} serve dual roles in the cellular response to genotoxic stress. On one hand, as checkpoints sensors they participate in the activation of the checkpoint signal transduction cascade and on the other hand, they directly participate in the recovery by stimulating BER enzymes. Several mechanisms could account for the observed physical and functional interactions of

the 9-1-1 clamp with the components of BER pathway. Firstly, the 9-1-1 complex and the Rad17- RF-C_{2-5} complex may attract different BER factors to the damage sites thus providing a structural framework for DNA repair machinery. Secondly, the checkpoint clamp/clamp loader pair may exert their stimulatory effect on the enzymatic activities of BER proteins via simple protein-protein interaction. Thirdly, both complexes may serve as a binding partner of the repair proteins. Taken together, the findings described in this review directly connect the DNA damage response with DNA repair, suggesting that the 9-1-1 complex might act as a core component of this connection.

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Table1. Functional and physical interactions between the 9-1-1 complex and the Rad17-RF-C_{2-5} complex with components of the LP-BER machinery

Checkpoint sensor	LP-BER component	Interaction and functional consequence	Reference
9-1-1	MutY DNA glycolase	Stimulation of glycosylase activity *In vitro* and *vivo* interaction	(43,44)
9-1-1	APE1	Stimulation of AP endonuclease activity *In vitro* and *vivo interaction*	(49)
Rad17-RF-C_{2-5}	APE1	*In vitro* and *vivo* interaction	*
9-1-1	Pol β	Stimulation of polymerase and strand displacement activities *In vitro* and *vivo* interaction	(50)
9-1-1	Fen 1	Stimulation of endo- and exonuclease activities *In vitro* and *vivo interaction*	(51,52)
9-1-1/Rad17-RF-C_{2-5}	Lig I	Stimulation of ligase activity *In vitro* and *vivo* interaction	(54-56)

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6.1 The checkpoint clamp, Rad9-Rad1-Hus1 complex, preferentially stimulates the activity of apurinic/apyrimidinic endonuclease 1 and DNA polymerase β in long patch base excision repair.

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The checkpoint clamp, Rad9-Rad1-Hus1 complex, preferentially stimulates the activity of apurinic/apyrimidinic endonuclease 1 and DNA polymerase β in long patch base excision repair

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ABSTRACT

Growing evidence suggests that the Rad9-Rad1-Hus1 complex (the 9-1-1 complex), besides its functions in DNA damage sensing and signaling pathways, plays also a direct role in various DNA repair processes. Recent studies have demonstrated that the 9-1-1 complex physically and functionally interacts with several components of the base excision repair (BER) machinery namely DNA polymerase β (Pol β), flap endonuclease 1 (Fen 1), DNA ligase I (Lig I) and the MutY homologue of *Schizosaccharomyces pombe*. In this work, we found for the first time that the 9-1-1 complex interacts *in vitro* and *in vivo* with the apurinic/apyrimidinic endonuclease 1 (APE 1), an early component of BER, and can stimulate its AP-endonuclease activity. Moreover, we show that the 9-1-1 complex possesses a stimulatory effect on long patch base excision repair (LP-BER) reconstituted *in vitro*. The enhancement of LP-BER activity is due to the specific stimulation of the two early components of the repair machinery, namely APE 1 and Pol β, suggesting a hierarchy of interactions between the 9-1-1 complex and the BER proteins acting in the repairosome. Overall, our results indicate that the 9-1-1 complex is directly involved in LP-BER, thus providing a possible link between DNA damage checkpoints and BER.

INTRODUCTION

The mammalian genome suffers from many endogenous and exogenous insults that modify DNA leading to base loss or base alterations. It has been estimated that ~100 000 modifications occur daily in the DNA of a single cell (1). In order to remove damage and to maintain the integrity of the genome, different DNA repair pathways have evolved. The major repair pathway protecting cells against a single-base DNA damage is base excision repair (BER) (2), although other pathways such as nucleotide excision repair and methyltransferase-mediated error-free repair are also involved (3). BER is often initiated by a damage type specific DNA glycosylase that cleaves the *N*-glycosydic bond of the damaged base, leaving an apurinic/apyrimidinic site, also referred to as an abasic site or apurinic/apyrimidinic (AP) site (4). Subsequently, AP endonuclease 1 (APE 1) cleaves the sugar-phosphate backbone at the 5′-side of the AP site resulting in 3′-hydroxyl and 5′-deoxyribose phosphate (dRP) groups flanking a one-nucleotide gap (5). DNA polymerase β (Pol β) inserts a first nucleotide into the gap, leaving nicked DNA with a 5′-dRP flap (6). At this point, the repair can be accomplished via two different BER sub-pathways depending on the nature of the AP site (2). In case of regular AP sites, Pol β removes the 5′-dRP group through its associated dRP-lyase activity (7), and the resulting nick is sealed by the DNA ligase III/XRCC1 complex. This sub-pathway is referred to as short-patch BER (SP-BER). However, if the sugar group is oxidized or reduced, Pol β cannot remove the 5′-dRP moiety and repair proceeds through the alternate long-patch BER (LP-BER) sub-pathway involving removal and

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replacement of 2–10 nucleotides (3,8). It has been found that, in the LP-BER pathway, the collaboration of Pol δ/ε, proliferating cell nuclear antigen (PCNA), replication factor C (RF-C), and flap endonuclease 1 (Fen 1) can displace the strand 3′ to the nick and synthesize up to 10 nucleotides. The resulting flap is cut by Fen 1 and the final nick is sealed by Lig I (9–13). However, growing evidence indicates that Pol β is a key enzyme in BER, that not only initiates both sub-pathways (14) but can also perform efficient strand displacement via the 'hit and run' mechanism (4). In this model Pol β and Fen 1 act successively, followed by the action of Lig I.

DNA repair is coordinated with cell-cycle progression (1,15) and with DNA-damage checkpoints (16). DNA-damage checkpoint pathways are activated at specific points during the cell cycle when the integrity of DNA is examined before progression to the next cell-cycle phase is allowed. The checkpoint response is a signal transduction cascade, where sensor proteins detect a lesion in the double helix and stimulate several effectors through the activity of different protein kinases. This may lead to temporary cell cycle arrest, slowing down of DNA replication, changes in the cellular transcriptional program, chromatin remodeling, induction of DNA repair genes, and occasionally to apoptosis (17,18). Some of the major checkpoint proteins namely ataxia telangectasia mutated protein (ATM), ATM-related protein (ATR), ATR interacting protein (ATRIP), Rad17, Rad9, Rad1, and Hus1, are thought to be involved in sensing and triggering DNA repair processes. Among those, the three human proteins Rad9, Hus1, and Rad1 form a heterotrimeric complex (the 9-1-1 complex) which exhibits structural similarity with the homotrimeric clamp formed by PCNA (19–21). The 9-1-1 complex has been characterized as a sensor of DNA damage and is targeted to damage sites following genotoxic stress (17,22–27). In addition, Rad17 associates with the four small subunits of the heteropentameric RF-C complex in a manner similar to the RF-C complex (20,21,28–30). Several studies showed that the 9-1-1 complex and Rad17-RF-C_{2-5} function as a clamp/clamp–loader pair, similarly to PCNA and RF-C (22,23,27,31,32). Moreover, the 9-1-1 complex, Rad17-RF-C_{2-5} and PCNA co-localize in foci formed upon DNA damage (22,33,34). These data suggested a mechanism in which Rad17- RF-C_{2-5} would recognize DNA lesions, allowing the recruitment of the 9-1-1 complex to those sites. ATM and ATR kinases are recruited simultaneously to the same sites of DNA damage but in a 9-1-1 complex and Rad17-RFC_{2-5} independent manner (35,36). The current model suggests that the 9-1-1 complex may facilitate the recruitment of the checkpoint effector kinase Chk1 (24,26) that is subsequently phosphorylated by the ATR/ATM kinases (17). Additionally, it has been recently proposed by two different groups, that the 9-1-1 complex and the Rad17–RFC_{2-5} alternative clamp loader could stabilize stalled replication forks (23,27).

Recently several new links between DNA damage checkpoints and various DNA repair processes have been discovered (34,37,38). Moreover, recent investigations showed several possible connections between the human 9-1-1 complex and the BER pathway (Table 1). First, a physical and functional interaction of the 9-1-1 complex with Pol β revealed that the 9-1-1 complex has a specific stimulatory effect on Pol β activity (39). Pol β stimulation resulted in an increase in its affinity for the primer-template. Interaction with the 9-1-1 complex stimulated DNA strand displacement synthesis raising the possibility that the 9-1-1 complex might attract Pol β to DNA-damage sites, thus connecting directly checkpoints and DNA repair. Very recently, we showed that the 9-1-1 complex interacts with and stimulates Lig I (40,41). In addition, similar physical and functional interactions with the 9-1-1 complex were identified for Fen 1 (42,43) and for the MutY DNA glycosylase homolog (44,45). In summary, these results suggested that the 9-1-1 complex could act as a recruiting platform for different factors involved in LP-BER.

Table 1. Functional consequences of the 9-1-1 complex interactions with components of the LP-BER machinery

LP-BER component	Functional consequence	References
MutY DNA glycosylase	Stimulation of glycosylase activity	(44,45)
APE 1	Stimulation of AP endonuclease activity	Present study
Pol β	Stimulation of polymerase and strand displacement activities	(39)
Fen 1	Stimulation of endo- and exonuclease activities	(42,43)
Lig I	Stimulation of ligase activity	(40,41)

In order to get more insight into the functional consequences of these interactions, we have reconstituted LP-BER *in vitro* by using a duplex oligonucleotide substrate containing a lesion (a tetrahydrofuran (THF) moiety) that mimics a reduced AP site and the four human proteins APE 1, Pol β, Fen 1, and Lig I. Our results suggest for the first time that the 9-1-1 complex specifically stimulates the endonuclease activity of APE 1 in a single-enzyme assay as well as in the reconstituted LP-BER *in vitro*. Further analysis revealed that the 9-1-1 complex physically interacts with APE 1 *in vitro* as well as *in vivo*. We also present evidence that the 9-1-1 complex specifically stimulates LP-BER *in vitro* through the stimulation of strand displacement activity of Pol β. Importantly, under conditions applied in the reconstituted LP-BER assay, we could not detect any effect of the 9-1-1 complex on the enzymatic activities of Fen 1 and Lig I. Our data suggest that in a reconstituted LP-BER *in vitro* system, a hierarchy of interactions between the 9-1-1 complex and the components of the LP-BER repairosome exists.

MATERIALS AND METHODS

Chemicals

[γ-^{32}P]ATP (3000 Ci/mmol), [α-^{32}P]dTTP (3000 Ci/mmol) and Sephadex G-25 spin column were from Amersham Biosciences; unlabeled dNTPs were from Roche Molecular Biochemicals. Oligonucleotides were from Microsynth (Switzerland). The THF (dSpacer) was from

Glen Research. All other reagents were of analytical grade and were purchased from Merck or Fluka.

Nucleic acid substrates

The 100 mer oligonucleotide containing the synthetic (THF) AP site as well as the complementary strand and the corresponding primers were chemically synthesized and purified on denaturing polyacrylamide gel. The sequence of 100 mer with THF moiety is as follows: 5′ATCCTGATTGCTATCTGAATATGGTGGTGGTG GGCGCCGGCG(**X**/**G**)TGTGAATTCGGCACTGGCC GTCATCGTGATCTATCTTACAGTATGCTCTTGGT TGTA3′

In brackets, the position of the lesion located 43 from the 5′ end or the corresponding G residue in the undamaged template is indicated in bold letters (X, THF moiety). This double stranded substrate was used for the single enzyme assays as well as for the reconstituted LP-BER *in vitro*. Alternatively the ends of the substrate were blocked with biotin by annealing a THF moiety-containing strand to the complementary one, containing biotin at the 3′ and 5′ end respectively. For the pol β assay the substrate contained an additional 1 nucleotide gap; for the Fen 1 assay a 10 nt flap (5′-ATCTGATCGC) and for Lig I a nick. The structures of these substrates are depicted in Figure 1B.

Proteins and antibodies

Human AP endonuclease 1 was obtained from Enzymax LLC. Bovine serum albumin (BSA) was purchased from New England BioLabs. The 9-1-1 complex was isolated by co-expressing in Sf21 cells the three baculoviruses encoding the recombinant hRad1, his-hRad9, and hHus1. The complex was subsequently purified as previously described (39). Human PCNA was produced in *Escherichia coli* using the plasmid pT7/hPCNA and purified to homogeneity as described (46). Human recombinant Pol β, Fen 1, and Lig I were expressed in *E. coli* and purified as previously described (47–49). The anti-Hus1 and anti-Rad9 antibodies described in Toueille et al. (39) were a gift of R. Freire (Teneriffe, Spain). The goat anti-Rad1 antibody (N18), as well as the rabbit anti-APE 1 (Anti-Ref1, H300) antibody were from Santa Cruz biotechnology.

Enzymatic assays

APE 1 incision assay. A 100 bp duplex oligonucleotide substrate was prepared as follows: a 100- mer oligonucleotide containing THF moiety at the position 43 was annealed to the complementary strand (Figure 1B). Prior to annealing a lesion containing strand was 5′-end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Unincorporated labeled nucleotides were removed on a Sephadex G-25 spin column. APE 1 incision reactions were carried out in a reaction mixture (10 μl) containing 45 mM Hepes/KOH (pH 7.8), 70 mM KCl, 7.5 mM $MgCl_2$, 2 mM dithiothreitol, 0.5mM EDTA, 0.1mg/ml BSA, 2 mM ATP, 50 fmol of oligonucleotide substrate, and the indicated amounts of APE 1. The ends of the oligonucleotide substrate were either free or blocked with biotin as indicated in the Figure legends. Reactions were incubated at 37°C for 20 min and stopped by adding an equal volume of gel loading buffer (95% formamide, 20 mM EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanol). Following incubation at 100°C for 5 min, the reaction products were separated by electrophoresis on a 10% denaturing polyacrylamide gel and visualized by autoradiography. To determine the effect of the 9-1-1 complex on APE 1 activity, the reactions were performed as described above, with the exception that constant amounts of APE 1 were included in the reaction mixture and increasing amount of the 9-1-1 complex were added as indicated in Figure 4A.

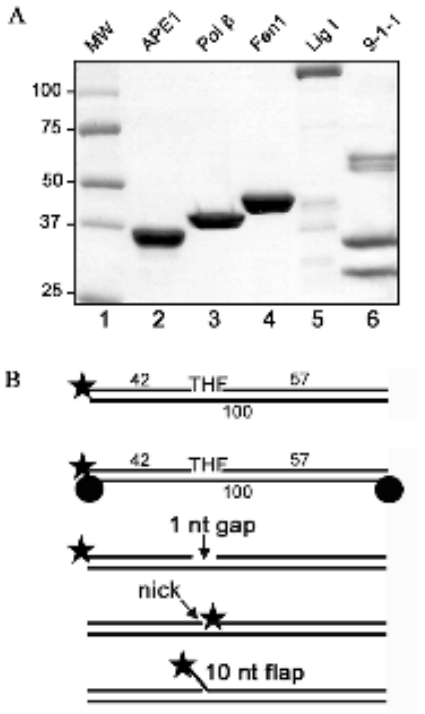

Figure 1. Proteins and substrates used in reconstitution of long patch base excision repair *in vitro*. **(A)** Recombinant proteins were purified as described in 'Materials and Methods' and separated on a 8–20% gradient SDS-PAGE gel, and stained with Coomassie Blue. Lane 1: molecular weight markers; lane 2: APE 1 (2 μg); lane 3: Pol β (2 μg); lane 4: Fen 1 (2 μg); lane 5: Lig I (2 μg); lane 6: 9-1-1 complex (6 μg). **(B)** Schematic representation of the ^{32}P-5′-labeled oligonucleotide substrates used in the study: a 100 bp duplex oligonucleotide containing a THF moiety at the position 43 was used for repair reactions, the ends of the substrate were either free (unblocked substrate) or blocked with a biotin at each end (blocked substrate); a 100 bp duplex oligonucleotide with a 1 nucleotide gap at the same position was used for the Pol β assay; with a nick for the Lig I assay and with a 10 nucleotide flap for the Fen 1 reaction

Pol β assay. A 42 mer oligonucleotide was radiolabeled at 5′ end and annealed with a 57 mer downstream oligonucleotide to the complementary 100 mer strand.

The resulting ^{32}P-labeled duplex oligonucleotide has a 1-nt gap at the position 43 (Figure 1B). Pol β DNA synthesis was determined by measuring nucleotide insertion into the DNA substrate described above. Reactions were carried out in a final volume of 10 µl containing 45 mM Hepes/KOH (pH 7.8), 70 mM KCl, 7.5 mM $MgCl_2$, 2mM dithiothreitol, 0.5mM EDTA, 0.1mg/ml BSA, 2 mM ATP, 20 µM each of dATP, dCTP, dGTP, dTTP, 50 fmol of gapped oligonucleotide substrate, and the indicated amounts of Pol β. Reactions were incubated at 37°C for 20 min and stopped by adding an equal volume of gel loading buffer (95% formamide, 20 mM EDTA, 0.02% bromophenol blue,and 0.02% xylene cyanol). Following incubation at 100°C for 5 min, the reaction products were separated by electrophoresis on a 10% denaturing polyacrylamide gel and visualized by autoradiography.

Pol β activity assay on the lesion-containing substrate. Pol β activity on the ^{32}P-5′-labeled, duplex oligonucleotide substrate containing THF moiety at the position 43 was assayed in the presence of a constant amount of APE 1. Reactions were incubated for 20 min at 37°C in the reaction buffer (10 µl) that contained 45 mM Hepes/KOH (pH 7.8), 70 mM KCl, 7.5 mM $MgCl_2$, 2 mM dithiothreitol, 0.5 mM EDTA, 0.1mg/ml BSA, 2 mM ATP, 20 µM each of dATP, dCTP, dGTP, dTTP, 50 fmol of THF containing oligonucleotide substrate, 55.5 fmol of APE 1, and the indicated amounts of Pol β. Following incubation, reactions were stopped by adding an equal volume of gel loading buffer (95% formamide, 20 mM EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanol), heated at 100°C for 5 min, and the products were separated by electrophoresis on a 10% denaturing polyacrylamide gel and visualized by autoradiography.

Fen 1 Endonuclease Assay. The Fen 1 assay was performed as described with some modifications (42). To prepare a flap substrate, 42 and 68 mer oligonucleotides were annealed to the complementary 100 mer strand. Prior to annealing a 68 mer oligonucleotide was radiolabeled at the 3′ end with terminal deoxynucleotidyltransferase and [α-^{32}P]ddATP. A ^{32}P-Labeled duplex oligonucleotide, thus prepared, had a 10-nt flap at the position 43 (Figure 1B). The Fen 1 cleavage assay was carried out in the reaction mixture (10 µl) containing 45 mM Hepes/KOH (pH 7.8), 70 mM KCl, 7.5 mM $MgCl_2$, 2 mM dithiothreitol, 0.5 mM EDTA, 0.1 mg/ml BSA, 2 mM ATP, 50 fmol of flap oligonucleotide substrate and, the indicated amounts of Fen 1. Reactions were incubated at 37°C for 20 min and stopped by adding an equal volume of gel loading buffer (95% formamide, 20 mM EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanol). Following incubation at 100°C for 5 min, the reaction products were separated by electrophoresis on a 10% denaturing polyacrylamide gel and visualized by autoradiography. In order to measure the stimulatory effect of the 9-1-1 complex on Fen 1 endonuclease activity the reactions were performed under the same conditions, except that constant amounts of Fen 1 were present in the reaction buffer (25 fmol) and the indicated amounts of the 9-1-1 complex were added as indicated in Figure 8C.

Lig I Assay. The Lig I assay was performed as described with modifications (40) by using a 42 mer and ^{32}P-5′-labeled 58 mer oligonucleotides annealed to the complementary 100 mer strand. Reaction mixtures (10 µl) contained 45 mM Hepes/KOH (pH 7.8), 70 mM KCl, 7.5 mM $MgCl_2$, 2 mM dithiothreitol, 0.5 mM EDTA, 0.1 mg/ml BSA, 2 mM ATP, 50 fmol of a nicked oligonucleotide substrate, and the indicated amounts of Lig I. Reactions were incubated at 37°C for 20 min and stopped by adding an equal volume of gel loading buffer (95% formamide, 20 mM EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanol). Following incubation at 100°C for 5 min, the reaction products were separated by electrophoresis on a 10% denaturing polyacrylamide gel and visualized by autoradiography. To measure the stimulatory effect of the 9-1-1 complex on Lig I activity the reactions were performed under the same conditions except that constant amounts of Lig I were present in the reaction buffer (0.5 fmol) and the indicated amounts of the 9-1-1 complex were added as indicated in Figure 8D.

Reconstituted in vitro LP-BER. The LP-BER assay was performed as described previously with modifications (14,50). The complete repair reactions were carried out in the reaction buffer (10 µl) that included: 45 mM Hepes/KOH (pH 7.8), 70 mM KCl, 7.5 mM $MgCl_2$, 2 mM dithiothreitol, 0.5 mM EDTA, 0.1 mg/ml BSA, 2 mM ATP, 20 µM each o dATP, dCTP, dGTP, dTTP, and 50 fmol of ^{32}P-5′-labeled 100 bp duplex oligonucleotide substrate containing a THF moiety at the position 43. The ends of the oligonucleotide substrate were either free or blocked with biotin as indicated in the Figure legends. For the reconstitution of LP-BER using unlabeled substrate the same conditions were used with the exception that the concentration of dTTP was reduced to 8 µM and [α-^{32}P] dTTP (2.5 µCi) was added to the reaction mixtures. The reactions were initiated by the addition of purified APE 1 (55 fmol), Pol β (64 fmol), Fen 1 (93 fmol), and Lig I (245 fmol). Increasing amounts of the 9-1-1 complex were added to the reconstituted LP-BER *in vitro* reaction under limiting conditions for each of the four enzymes APE 1, Pol β, Fen 1, and Lig I (see 'Results'). After incubation at 37°C for 20 min, the reactions were stopped by adding an equal volume of gel loading buffer (95% formamide, 20 mM EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanol), heated at 100°C for 5 min, and the final products were separated by electrophoresis on a 10% denaturing polyacrylamide gel and visualized by autoradiography.

Pulldown assays. For the His-pulldowns, 6 µg of the his-tagged 9-1-1 complex or the his-tagged subunits Rad9, Rad1 or Hus1 were incubated with 1 µg APE 1 for 2 h at 4°C in pulldown buffer [50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.05% (v/v) NP-40]. Ni^{2+} beads, previously coated by overnight incubation in

pulldown buffer containing 5 mg/ml BSA, followed by three washes in pulldown buffer, were then added to the proteins. The samples were subsequently incubated at 4°C for 1 h. After washing five times in pulldown buffer containing 5 mM imidazole, the beads were heated for 5 min at 95°C in Laemmli buffer and the co-precipitated proteins were analyzed by western blot using the corresponding antibodies according to established methods.

For the APE 1-sepharose pulldowns, purified APE 1 or BSA, used as a negative control, was covalently coupled to CNBr-activated Sepharose (Amersham Pharmacia Biotech) as described by the supplier, at a final ratio of 1 μg of protein/μl of beads. APE 1-sepharose or BSA-sepharose (10 μl) were subsequently washed once in pulldown buffer containing 10 mg/ml BSA and three times in pulldown buffer. The beads were subsequently incubated with 300 ng of his9-1-1 complex for 2 h in pulldown buffer at 4°C. After washing five times in pulldown buffer, the beads were heated for 5 min at 95°C in Laemmli buffer and the co-precipitated proteins were analyzed by western blot using the corresponding antibodies according to established methods.

Whole cell extracts. For preparing total cell extracts, 7×10^6 293T cells were harvested by trypsinisation followed by centrifugation. The cell pellet was subsequently lysed in cell lysis buffer [50 mM Hepes-KOH, pH 7.5, 400 mM NaCl, 1 mM DTT, 2.5 mM $MgCl_2$, 20% glycerol, 0.5% (v/v) NP40, 2 μg/ml leupetpin, 1 μg/ml bestatin, 1 μg/ml pepstatin, 2 mM PMSF, 10 mM glycerophosphate, 1 mM NaF, 10 mM $Na_4P_2O_7$] for 15 min at 4°C. The cell lysate was subsequently centrifuged for 15 min at 10 000 rpm, the supernatant was collected and kept as a total cell extract. The protein concentration was determined by using the Bradford assay.

Immunoprecipitations. For immunoprecipitations, 25 μl of protein G sepharose were coated for 3 hours at 4°C with 100 μg BSA in IP buffer (40 mM Hepes-KOH, pH 7.5, 100 mM NaCl, 8 mM $MgCl_2$, 2 μg/ml leupetpin, 1 μg/ml bestatin, 1 μg/ml pepstatin, 2 mM PMSF, 10 mM glycerophosphate, 1mM NaF, 10 mM $Na_4P_2O_7$) and subsequently incubated overnight with 3 μg of anti-Rad9 antibody or the unimmunized rabbit IgG. After three washes in IP buffer, the beads were incubated with 1mg of 293 T total cell extract for 3 h at 4°C. After incubation, the beads were washed three times in IP buffer containing 0.05% NP40 and subsequently heated for 5 min at 95°C in Laemmli buffer. The co-precipitated proteins were analyzed by western blot using the corresponding antibodies according to established methods.

RESULTS

Proteins and substrates used in this study and their requirements in long patch base excision repair

The 9-1-1 complex has been shown to interact and stimulate the activity of several LP-BER factors *in vitro* (Table 1). Therefore, our aim was to determine if the 9-1-1 complex could also stimulate the complete repair of an abasic site through the stimulation of different components of LP-BER. To address this question, we first reconstituted LP-BER *in vitro* using a set of the four different protein components APE 1, Polβ, Fen 1, and Lig I, all of which were highly purified from recombinant sources (Figure 1A). First they were tested for their enzymatic activities as described in 'Material and Methods'. As a substrate for all repair reactions we used a 100 bp oligonucleotide duplex containing a THF moiety at the position 43, which resembles a reduced abasic site (AP site) (Figure 1B). The requirement of the different components of LP-BER was tested as shown in Figure 2B. In this experiment we used a THF-containing substrate labeled at its 5′ end. For this reason the substrate (Figure 2 lane 8) and the product of the reaction (Figure 2 lane 7) showed the same migration. However, when deoxyribonucleotides or Pol β were absent from the reaction (lane 2 and 4, respectively) the product corresponded to the DNA substrate cleaved by APE 1. Subsequently, the reaction performed in the absence of Fen 1 showed an efficient strand displacement synthesis performed by Pol β (Figure 2 lane 5). However, the resulting flap-structure intermediates could not be ligated by Lig I to generate the fully repaired product. When Lig I was excluded from the reaction mixture, Pol β synthesized up to 6 nucleotides, which led to the generation of the flap structure, subsequently removed by Fen 1 cleavage (Figure 2B, lane 6). This limited DNA synthesis (compare lanes 5 and 6) reflects the effect of Fen 1 on Pol β strand displacement synthesis, illustrating the previously described so-called 'hit and run' mechanism (4). Surprisingly, incubation of the substrate with all four protein components but without addition of ATP resulted in the complete repair of the damaged strand even though ATP is known to be required for Lig I activity (Figure 2B, lane 1). The explanation for this observation is that a fraction of Lig I used in this assay might have been purified in its pre-adenylated form, thus allowing ligation to occur in the absence of ATP. As expected, omission of APE 1 resulted in the absence of cleavage and of the subsequent LP-BER reaction. This results show that the reconstituted LP-BER reaction is dependent on the presence and proper activity of all required enzymatic components, hence demonstrating that all the steps of the reconstituted reaction are functional. Incubation of the oligonucleotide substrate in the reaction buffer alone (Figure 2B, lane 8) or with addition of the maximal amount of the 9-1-1 complex used in the study, did not show any enzymatic contamination of the purified 9-1-1 checkpoint clamp.

Fine-tuning of the different steps of long patch base excision repair *in vitro*

Next, in order to study the effect of the 9-1-1 complex on the LP-BER machinery, each protein component was titrated against all the others (Figure 3). This allowed us to determine the amount of enzyme that significantly

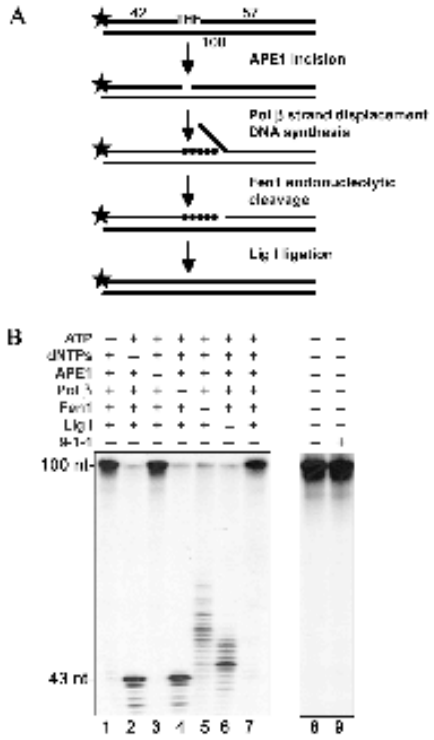

Figure 2. Protein requirements for the long patch base excision repair *in vitro* system. **(A)** Schematic representation of the ^{32}P-5′-labeled oligonucleotide substrates used. **(B)** The LP-BER *in vitro* reaction was performed as described in 'Materials and Methods'. The complete reaction contained a 100 bp duplex oligonucleotide (50 fmol) with a THF moiety at the position 43, APE 1 (55 fmol), Pol β (64 fmol), Fen 1 (93 fmol) and Lig I (245 fmol). Lane 1: ATP omitted; Lane 2: dNTPs omitted; Lane 3: APE 1 omitted; lane 4: Pol β omitted; lane 5: Fen 1 omitted; lane 6: Lig I omitted; lane 7: complete reaction; lane 8: the 100 bp duplex oligonucleotide (50 fmol) was incubated in the reaction buffer alone and (lane 9) with 1 pmol of the 9-1-1 complex. After incubation reactions were stopped by adding an equal volume of formamide-dye solution and products were analyzed on a 10% denaturing polyacrylamide gel.

reduces repair at any given step, therefore serving as a limiting factor of the total reaction. First, the repair was performed by adding increasing amounts of APE 1 to the reaction mixture, which contained saturating amounts of Pol β, Fen 1 and Lig I (Figure 3A). In order to avoid the problem of the substrate and the BER reaction product running at the same position on the gel, the substrate remained unlabeled in this experiment, and the repair was visualized by the incorporation of [α-^{32}P] dTTP, the first nucleotide incorporated opposite A after the lesion. The minimal amount of APE 1 that incised the damaged strand and allowed repair to take place although at a low level, was 14-fold lower than the amount of enzyme required for the complete repair (Figure 3A, compare 5 and 55 fmols). Incubation of 5′-^{32}P labeled substrate with

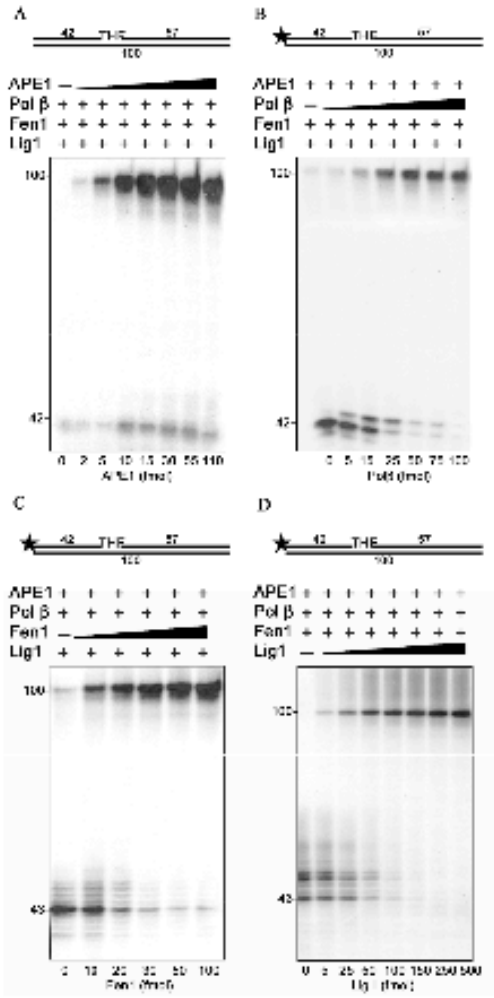

Figure 3. Fine-tuning of the different enzymes in the long patch base excision repair *in vitro*. The LP-BER *in vitro* reaction was performed as described in 'Materials and Methods'. All reactions were stopped by adding an equal volume of formamide-dye solution and products were analyzed on a 10% denaturing polyacrylamide gel. **(A)** The reaction mixtures (10 μl) contained (besides all components described in 'Materials and Methods') unlabeled 100 bp duplex oligonucleotide (50 fmol), [α-^{32}P] dTTP (2.5 μCi), Pol β (64 fmol), Fen 1 (93 fmol), Lig I (245 fmol) and the indicated amounts of APE 1. Reactions were incubated for 20 min at 37°C. **(B)** The reaction mixtures (10 μl) contained (besides all components described in 'Materials and Methods') ^{32}P-5′-labeled 100 bp duplex oligonucleotide (50 fmol), APE 1 (55 fmol), Fen 1 (93 fmol) and Lig I (245 fmol). Reactions were incubated for 20 min at 37°C with the indicated amounts of Pol β. **(C)** The reaction mixtures (10 μl) contained (besides all components described in 'Materials and Methods') ^{32}P-5′-labeled 100 bp duplex oligonucleotide (50 fmol), APE 1 (55 fmol), Pol β (64 fmol), Lig I (245 fmol) and increasing amounts of Fen 1. Reactions were incubated for 20 min at 37°C. **(D)** The reaction mixtures (1 μl) contained (besides all components described in 'Materials and Methods') ^{32}P-5′-labeled 100 bp duplex oligonucleotide (50 fmol), of APE 1 (55 fmol) of Pol β (64 fmol), Fen 1 (93 fmol) and indicated amounts of Lig I. Reactions were incubated for 20 min at 37°C.

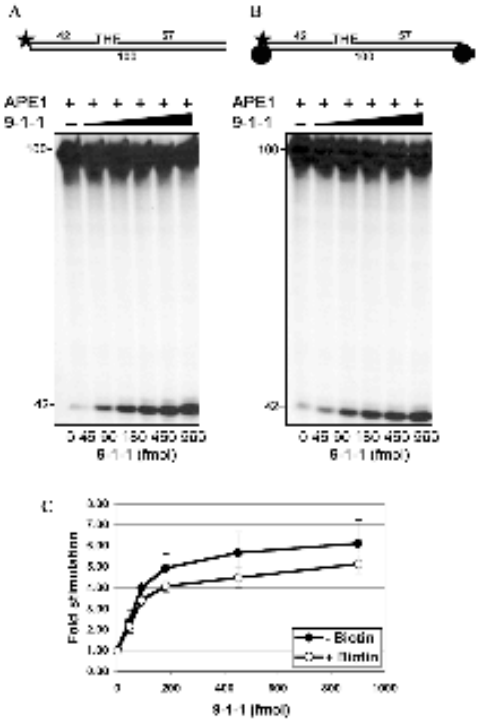

Figure 4. The 9-1-1 complex specifically stimulates the endonuclease activity of APE 1 *in vitro*. The APE 1 incision assay was performed as described in 'Materials and Methods'. Reactions were stopped by adding an equal volume of formamide-dye solution and products were analyzed on a 10% denaturing polyacrylamide gel. **(A)** An APE 1 reaction mixture (10 µl) contained (besides all components described in 'Materials and Methods') ^{32}P-5′-labeled 100 bp duplex oligonucleotide (50 fmol see Figure 1B), APE 1 (2 fmol). Reactions were incubated for 20 min at 37°C with the indicated amounts of the 9-1-1 complex. **(B)** As A but with the blocked substrate (50 fmol). **(C)** Quantification of the stimulation of APE 1 endonuclease cleavage by the 9-1-1 complex on the substrate with free ends (closed circles) and with the ends blocked with biotin (open circles). The values represent the mean of three independent experiments. The error bars correspond to the standard error of the mean.

saturating amounts of APE 1, Fen 1, and Lig I and increasing amounts of Pol β then allowed determining the amount of enzyme performing a limited strand displacement DNA synthesis (15 fmol, Figure 3B). That was approximately 4-fold less as compared with the amount of Pol β used to obtain complete repair (compare to Figure 3B, 50 fmols). To determine the fine-tuning of Fen 1 and Lig1 activities in LP-BER, both enzymes were titrated in the presence of 5′-^{32}P labeled substrate. All the proteins were present in saturating amounts in the reaction mixture except of Fen 1 (Figure 3C) and Lig I (Figure 3D), respectively. For further experiments we chose amounts of Fen 1 (Figure 3C, compare 20 and 100 fmols) and Lig I (Figure 3D, compare 25 and 250 fmols) that were 10-fold decreased as compared with the level required for the complete LP-BER.

The Rad9-Rad1-Hus1 complex specifically stimulates the endonuclease activity of APE 1 without encircling the DNA substrate

Previous studies have shown that the 9-1-1 complex stimulates the activities of Pol β, Fen 1, and Lig I on the substrates that mimic transient intermediates of the respective steps of LP-BER. We sought to determine whether the 9-1-1 complex exerts the same effect on the activity of APE 1. Incubation of 5′-^{32}P labeled, THF-containing substrate with APE 1 and increasing amounts of the 9-1-1 complex led to a 6-fold stimulation of the APE 1 endonuclease activity, as shown by the accumulation of APE 1 cleavage product (42 nucleotides) (Figure 4A, 45 and 450 fmols and Figure 4C). Moreover, in control experiments this stimulation was not observed when the substrate and APE 1 were incubated with increasing amounts of either PCNA or BSA, thus confirming the specificity of this stimulation (data not shown). Next we tested whether the loading of the 9-1-1 complex onto DNA is necessary for the observed stimulatory effect. It has been shown that 9-1-1 complex can be loaded onto DNA *in vitro* in a Rad17-RFC$_{(2-5)}$ dependent manner (22,23,27,31,32). In order to determine if the 9-1-1 complex has to encircle DNA to stimulate APE 1 activity, we used a THF-containing substrate with both ends blocked with biotin. Efficient blockage of the ends was checked in a pol δ stimulation assay in the presence of PCNA, and resulted in a complete abolishment of PCNA dependent pol δ activity (data not shown). As shown in Figure 4B, incubation of 5′-^{32}P labeled, blocked substrate with APE 1 and increasing amounts of the 9-1-1 complex (Figure 4B, 45 and 900 fmols) led to a similar extent of APE 1 stimulation as compared with the reactions performed in the presence of unblocked substrate (Figure 4C). Thus, we concluded that the loading of the checkpoint clamp onto DNA is not required to stimulate APE 1 endonuclease cleavage *in vitro*.

The 9-1-1 complex physically interacts with APE 1 *in vitro* and *in vivo*

Next, in order to assess the relevance of the observed stimulation of APE 1 by the 9-1-1 complex, we investigated the physical interaction of the two proteins. We first performed pulldown experiments in order to determine whether the two proteins interact specifically and directly *in vitro* (Figure 5A). By incubation of APE 1-bound sepharose beads with purified 9-1-1 complex, we were able to show that the 9-1-1 complex was interacting with APE 1 as Rad9, Rad1 and Hus1 were detected in the pulled-down fraction (Figure 5A, left panel). Next, to confirm these data, his-9-1-1 complex was incubated together with APE 1 followed by binding of the 9-1-1 complex to Ni^{2+} beads via the his-Rad9 subunit (Figure 5A, right panel). We were first able to show that the three subunits hisRad9, Rad1, and Hus1, indeed form a complex under those conditions, as the three subunits were co-precipitated with the Ni^{2+} beads in the presence or absence of APE 1 (Figure 5A, right panel and data not shown), although only Rad9 possessed a his-tag. In this pulldown, APE 1 was also co-precipitated with the

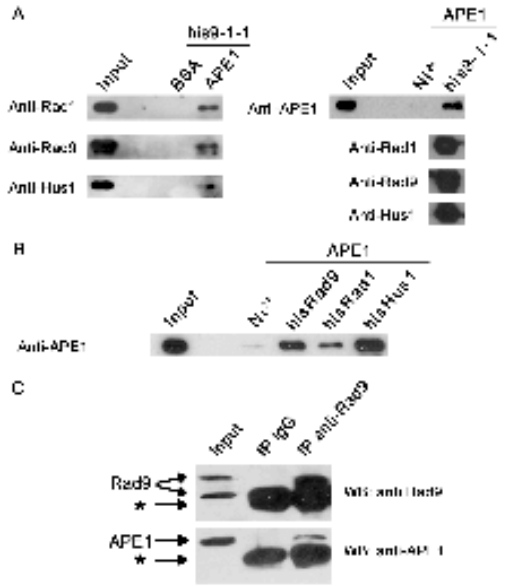

Figure 5. The 9-1-1 complex physically interacts with APE 1 *in vitro* and *in vivo*. **(A)** Direct interaction of the 9-1-1 complex with APE 1 *in vitro*. APE 1-sepharose pulldowns (right panel) were performed by incubating APE 1-sepharose beads, or BSA-sepharose as a negative control, together with purified his9-1-1 complex as described in 'Materials and Methods'. His9-1-1 pulldowns (left panel) were performed by incubating purified his9-1-1 complex with purified APE 1 and subsequent binding to Ni^{2+} beads. Five percent of the pulldown were used to check the presence of all the subunits of the 9-1-1 complex (his-Rad9, Rad1 and Hus1), and the remaining sample was used to detect co-precipitated APE 1 by SDS-PAGE followed by western blot analysis. Input represents 2% of the total amount of interacting protein used in the pulldown experiments. **(B)** Physical interaction of APE 1 with the 9-1-1 complex subunits. His-pulldowns were performed by using the individual subunits his-Rad1, his-Rad9, and his-Hus1 as described in **A**. **(C)** The 9-1-1 complex interacts with APE1 *in vivo*. Immunoprecipitation experiments were performed as described in 'Material and Methods' by incubating 293T total cell extracts with anti-human Rad9 antibody. Presence of immuno-precipitated Rad9 and co-precipitated APE1 were analyzed by SDS-PAGE followed by western blot analysis. The lane IP IgG contains the control immunoprecipitation performed in the presence of un-immunized rabbit IgG. Input represents 5% of the amount of extract used for immunoprecipitation. Arrows indicate the positions of endogenous APE1 and Rad9. The bands indicated by asterisks correspond to the antibody heavy and light chain respectively.

9-1-1 complex. Hence, these results demonstrate for the first time a direct physical interaction of the 9-1-1 complex with APE 1.

We next investigated whether APE 1 was able to interact with all three subunits of the 9-1-1 complex, as previously shown for the other LP-BER proteins (39–43). To address this question we performed pulldown experiments using his-tagged Rad1, Rad9, or Hus1 incubated with APE 1 and subsequently bound to Ni^{2+} beads (Figure 5B). As revealed by western-blot against APE 1, APE 1 was able to interact with the three separated subunits of the 9-1-1 complex, although with a weaker intensity for Rad1.

Finally we tested whether the interaction of the 9-1-1 complex with APE 1 could also be detected in cell extracts, in order to confirm its *in vivo* relevance. Indeed by performing co-immunoprecipitation with an anti-human Rad9 antibody we were able to detect APE 1 in the immuno-precipitated fraction, whereas it was not detectable in the negative control performed with un-immunized rabbit IgG (Figure 5C, bottom). The immuno-precipitation of Rad9 was checked by detecting of one of its phosphorylated forms, known to be present in untreated cells (19), that migrated above the heavy IgG chains, whereas the main band corresponding to the non-phosphorylated form was not visible due to its migration at the same level than the heavy IgG chains (Figure 5C, top). In addition, the same experiment performed with HeLa cell extracts gave similar results (data not shown). We therefore concluded that the interaction of the 9-1-1 complex with APE 1 also occurs in human cells, thus supporting an *in vivo* role for this interaction.

The Rad9-Rad1-Hus1 complex specifically stimulates the endonuclease activity of APE 1 in LP-BER *in vitro*

Having established that APE 1 can be stimulated by the 9-1-1 complex via a direct interaction, an important question arose; namely if the 9-1-1 complex is able to enhance the activity of APE 1 directly in the reconstituted LP-BER reaction. To address this question we performed a LP-BER assay where APE 1 was the limiting factor of the reaction (Figure 6B, compare 4 and 70 fmols of APE 1). Indeed, addition of increasing amounts of the 9-1-1 complex to the reaction mixture enhanced the yield of repair up to 4-fold (Figure 6B, 45–450 fmols). In contrast, PCNA and BSA had no significant effect on the repair efficiency (Figure 4 C, D and E, respectively). In addition, we tested if the blockage of the substrate ends influenced the reported stimulation. We observed that the 9-1-1 complex was able to stimulate APE 1 activity without being loaded onto DNA in the reconstituted BER reaction, as well as in the single enzyme assay (Figure 6F). Thus, our results demonstrate for the first time that the 9-1-1 complex specifically stimulates LP-BER *in vitro* via stimulation of the endonucleolytic cleavage by APE 1.

The Rad9-Rad1-Hus1 complex enhances the strand displacement activity of Pol β in long patch base excision repair *in vitro*

Our next aim was to examine whether the 9-1-1 complex can influence LP-BER *in vitro* via stimulation of Pol β activity. We have previously shown that the 9-1-1 complex increases the affinity of Pol β for the primer-template and stimulates its DNA strand displacement activity (39). To test if the same effect could be observed in LP-BER, we performed a repair reaction using a 5′-^{32}P labeled, THF-containing substrate in the presence of limiting amounts of Pol β (Figure 7, compare 64 and 15 fmols of pol β). Addition of the 9-1-1 complex to the reaction resulted in approximately 3-fold stimulation of the Pol β strand displacement activity and therefore increased efficiency of

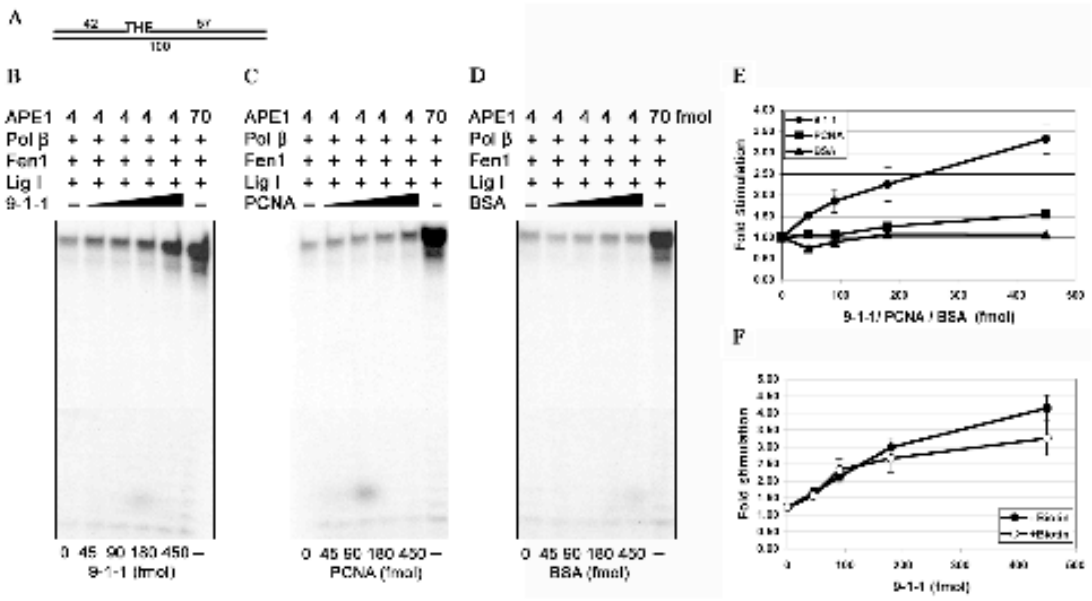

Figure 6. The 9-1-1 complex specifically stimulates the endonuclease activity of APE 1 in LP-BER *in vitro*. **(A)** Schematic representation of the substrate used in the LP-BER reaction. **(B)** LP-BER assay was performed as described in 'Materials and Methods' The reaction mixtures (10 μl) contained (besides all components described in 'Materials and Methods') unlabeled 100 bp duplex oligonucleotide (50 fmol), [α-^{32}P] dTTP (2.5 μCi), APE 1 (4 fmol), Pol β (64 fmol), Fen 1 (93 fmol), and Lig I (245 fmol). Reactions were incubated for 20 min at 37°C with the indicated amounts of the 9-1-1 complex, **(C)** as B but with the indicated amounts of PCNA **(D)** as B but with the indicated amounts of BSA. **(E)** Quantification of the stimulation of APE 1 activity in LP-BER *in vitro* by the 9-1-1 complex (circles); PCNA (rectangles) and BSA (triangles). The values represent the mean of three independent experiments. The error bars correspond to the standard error of the mean. **(F)** Quantification of the stimulation of APE 1 activity in LP-BER *in vitro* by the 9-1-1 complex, on the substrate with free ends (closed circles) and with the ends blocked with biotin (open circles). The values represent the mean of three independent experiments. The error bars correspond to the standard error of the mean.

the total repair. In contrast no effect was observed when PCNA and BSA were tested under the same conditions, which confirms the specificity of the reported stimulation (Figure 7B and C).

The Rad9-Rad1-Hus1 complex has no effect of the activities of Fen 1 and Lig I in long patch base excision repair *in vitro*

To further elucidate the involvement of the 9-1-1 complex in LP-BER we studied the effect of the checkpoint clamp on the activities of Fen 1 and Lig I in LP-BER *in vitro*. Similar to the previous experiments, we performed the LP-BER assay in the presence of limiting amounts of Fen 1 (Figure 8A) or Lig I (Figure 8B). Surprisingly, under those conditions, the 9-1-1 complex was unable to stimulate the enzymatic activities of Fen 1 and Lig I in LP-BER *in vitro*. However, the same 9-1-1 complex used in a single enzyme assay using DNA substrates representing the corresponding LP-BER intermediates was, as previously reported (40,42), able to stimulate enzymatic activities of Fen 1 and Lig I (Figure 8C and D). Thus, it appears that in the reconstituted LP-BER pathway, the 9-1-1 complex predominantly stimulates the enzymatic activities of those components of BER that act in the early steps of repair, such as APE 1 and Pol β, whereas the repair enzyme activities occurring during later steps, i.e. Fen 1 removal of the displaced strand or Lig I ligation, are not influenced directly by the checkpoint clamp in the presence of all the LP-BER proteins.

DISCUSSION

A growing amount of evidence indicates that the 9-1-1 complex, initially shown to be important for the DNA damage sensing and signaling pathways (17), plays also a more direct role in various DNA repair processes such as nucleotide excision repair (37), double strand break repair (34), translesion synthesis (34,37,38), and BER (Table 1). In previous reports we and others have demonstrated that the 9-1-1 complex physically and functionally interacts with several important components of the BER machinery namely Pol β (39), Fen 1 (42,43), Lig I (40,41), and the MutY DNA glycosylase homolog (MYH) (44,45). In this work we provide evidence that the 9-1-1 complex is directly involved in LP-BER. The evidence is derived from our demonstration that the 9-1-1 complex markedly stimulates a reconstituted LP-BER system *in vitro* and that this stimulation is due to the effect the 9-1-1 complex exerts on the enzymatic activities of the two early components of LP-BER namely APE 1 and Pol β.

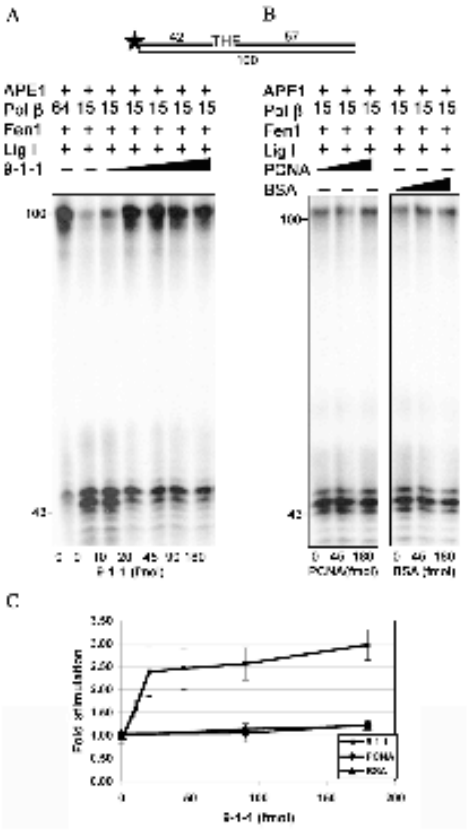

Figure 7. The 9-1-1 complex specifically stimulates the activity of Pol β in long patch base excision repair *in vitro*. The LP-BER *in vitro* reaction was performed as described in 'Materials and Methods'. Reactions were stopped by adding an equal volume of formamide-dye solution and products were analyzed on a 10% denaturing polyacrylamide gel. **(A)** The reaction mixtures (10 μl) contained (besides all components described in 'Materials and Methods') ^{32}P-5′-labeled 100 bp duplex oligonucleotide (50 fmol), APE 1(55 fmol), Pol β (15 fmol), Fen 1 (93 fmol), and Lig I (245 fmol). Reactions were incubated for 20 min at 37°C with increasing amounts of the 9-1-1 complex. **(B)** as A but with the indicated amounts of PCNA or BSA. **(C)** Quantification of the stimulation of Pol β activity in LP-BER *in vitro* by the 9-1-1 complex (rectangles); PCNA (rhomboids) and BSA (triangles). The error bars correspond to the standard error of the mean.

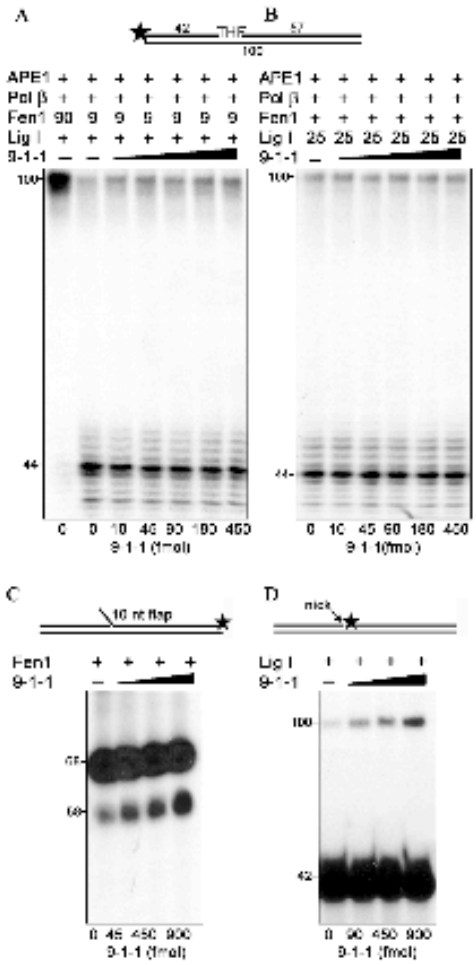

Figure 8. The 9-1-1 complex has no effect on the activities of Fen 1 and Lig I in long patch base excision repair *in vitro*. The LP-BER *in vitro* reaction was performed as described in 'Materials and Methods'. Reactions were stopped by adding an equal volume of formamide-dye solution and products were analyzed on a 10% denaturing polyacrylamide gel. **(A)** The reaction mixtures (10 μl) contained (besides all components described in Materials and Methods) ^{32}P-5′-labeled 100 bp duplex oligonucleotide (50 fmol), APE 1 (55 fmol), Pol β (64 fmol), Fen 1 (9 fmol), and of Lig I (245 fmol). Reactions were incubated for 20 min at 37°C with the indicated amounts of the 9-1-1 complex. **(B)** The reaction mixtures (10 μl) contained (besides all components described in Materials and Methods) ^{32}P-5′-labeled 100 bp duplex oligonucleotide (50 fmol), APE 1 (55 fmol), Pol β (64 fmol), Fen 1 (93 fmol), and Lig I (25 fmol). Reactions were incubated for 20 min at 37°C with the indicated amounts of the 9-1-1 complex. **(C)** A Fen 1 reaction mixture (10 μl) contained (besides all components described in Materials and Methods) a 10 nucleotide flap substrate (50 fmol, see Figure 1B) and Fen 1 (25 fmol). Reactions were incubated for 20 min at 37°C with the indicated amounts of the 9-1-1 complex. **(D)** A Lig I reaction mixture (10 μl) contained (besides all components described in Materials and Methods) a nicked oligonucleotide (50 fmol, see Figure 1B) and Lig I (0.5 fmol). Reactions were incubated for 20 min at 37°C with the indicated amounts of the 9-1-1 complex.

Interestingly, we showed for the first time that the 9-1-1 complex directly interacts with APE 1 *in vitro* and *in vivo*, and that the 9-1-1 complex specifically stimulates the APE 1 endonuclease activity on a THF-containing substrate independently of its loading onto DNA. However, it is well known that *in vivo*, APE 1 is a very abundant protein possessing multiple cellular functions. In addition to its role in BER, it is involved in oxidative DNA damage repair and stimulates the DNA binding activity of AP-1 (Fos, Jun) proteins as well as nuclear factor-κB (NF-κB), the polyoma virus enhancer-binding protein 2 (PEBP2), the early growth response-1 (Egr-1), Myb, members of the

ATF/CREB family, the hypoxia inducible factor-1α(HIF-1α), the HIF-like factor (HLF), Pax-5, and Pax-8 [reviewed in (51)]. APE 1 possesses also the major AP-1 redox activity in cells and represents a novel redox component of signal transduction cascades that regulates eukaryotic gene expression. In addition APE 1 has been implicated in the control of p53 activity through redox dependent and independent mechanisms. It has been also closely linked to apoptosis and altered levels of APE 1 have been found in some cancers. Therefore, APE 1 appears to form a link between the BER pathways, cancer, and regulation of transcription factors, oxidative signaling and cell cycle control. Hence, although APE 1 is known to be very abundant, some regulatory mechanisms must exist, that target this multifunctional enzyme in a timely and spatially regulated manner towards its required functions in specific cellular conditions. For this reason and considering our data, we hypothesize that the 9-1-1 complex may not simply stimulate APE 1 but it may also attract it to the sites of damage and target its functions towards the BER pathway. On the other hand, our efforts to determine the mechanism of stimulation of APE 1 by the 9-1-1 complex by biochemical ways remained still unsolved. First, the use of kinetic studies to address this point proved difficult, since the stimulatory effect of the 9-1-1 complex on APE1 appears to be of a complex nature and does not follow the classical rules of enzymology (e.g. reduction of the Km and/or increase of the Vmax). Second, in electro-mobility shift assays (EMSA) performed in the absence of Mg^{2+}, addition of the 9-1-1 complex did not lead to an increase in the binding of APE 1 to a double stranded THF-containing substrate (data not shown). However, the absence of divalent cation in this experiment might create a context in which the 9-1-1 complex can not exert its stimulatory effect on APE 1, thus making it impossible to conclude whether the 9-1-1 complex does not affect the binding of APE 1 to DNA at all, or only in the system we used. More importantly we showed in this manuscript that the 9-1-1 complex interacts *in vivo* with APE 1 in human cells, as it has been shown previously for other LP-BER enzymes (41, 43, 45), thus supporting a functional role for the interaction of the 9-1-1 complex with APE 1. In addition preliminary experiments performed in our group indicated that APE 1 and the 9-1-1 complex co-localize *in vivo* and that this effect is enhanced upon H_2O_2 and IR treatment (Gembka and Hübscher, unpublished data).

Moreover, we showed that in our *in vitro* system the 9-1-1 complex significantly stimulates Pol β strand displacement activity, which is in agreement with previously reported functional and physical interactions between these two proteins (39). However, we could not observe any effect of the 9-1-1 complex on the activities of Fen 1 and Lig I in the reconstituted LP-BER *in vitro*. At first sight, this suggests a strong discrepancy between our findings and previous reports indicating that the 9-1-1 complex stimulates the enzymatic activities of Fen 1 (42,43) and Lig I (40,41). However, we suggest that in our *in vitro* system a hierarchy of stimulation and protein–protein interactions between the 9-1-1 complex and the BER components exists. Thus, it seems to be reasonable to speculate that the 9-1-1 complex, which functions as a sensor of DNA damage, once localized to the damage site recruits other repair proteins where their functions are necessary. This is in agreement with the reported *in vivo* interaction studies showing that the 9-1-1 co-immunoprecipitates with nearly all components of LP-BER (this work, 41,43,45). However, at the site of lesion, the 9-1-1 complex may exert its stimulatory effect only on the enzymatic activities of the early components of the repair machinery e.g. APE 1 and Pol β. This effect seems to be no longer necessary at the latter steps of repair involving the action of Fen 1 and Lig I. However, those enzymes, acting late in the BER process, are known to be stimulated by PCNA whereas this has not been shown for Pol β and APE 1 so far. Hence, one might speculate that after a first step of recruitment of the early repair enzymes to the lesion by the 9-1-1 complex, PCNA might be responsible for completion of the pathway through its action on the late BER enzymes. This hypothesis is further supported by the fact that PCNA has also been shown to co-localize with DNA repair factories containing the 9-1-1 complex upon DNA damage (34). In addition the complete BER reaction involves several proteins that establish a complex network of physical and functional interactions with each other and with the 9-1-1 complex. An example of this is the recently demonstrated 'hit and run' mechanism that shows how Fen 1 influences Pol β (4). In this context, one can assume that the effect of the 9-1-1 complex can be abolished or masked by the influence of the other proteins present in the reaction, or that the complex might be trapped by one of the enzymes present in the reaction.

On the other hand we cannot exclude that the proposed model may differ from the *in vivo* situation. Another striking finding we and the others observed (41,43) using *in vitro* systems is that encircling of the DNA substrate by the 9-1-1 complex is not required to stimulate the activities of repair enzymes. This indicates a different mechanism than in case of the PCNA ring, which absolutely requires a loading process to further stimulate Fen 1 and Lig I activities. One explanation could be that the observed stimulatory effect results from the direct protein–protein interactions and does not involve the loading of the 9-1-1 complex in Rad17- RFC_{2-5} dependent manner. This is supported by the fact that APE 1, as well as Pol β, Fen 1, and the MutY DNA glycosylase also interact *in vitro* with the single subunits of the checkpoint clamp (this work, 39–43 for details). Still, *in vivo* only the loading of the 9-1-1 complex onto chromatin leads to the local increase of its concentration at the damage sites, which is very important for the functions of the 9-1-1 complex in DNA damage signaling and most probably, in DNA repair.

Moreover, the tumor suppressor p53 and its downstream target p21 are other important components of DNA damage response that are directly connected to BER (52–55). It has been speculated that at low level, p53 actively functions in BER whereas a high level of p53 supports a global DNA-damage response and, in cases of excessive genotoxic stress, apoptosis (52,53). During cellular stress p53 up-regulates the expression of p21, which in turn interacts with many other proteins involved

in replication, transcription, and signal transduction. In particular it binds to PCNA causing the inactivation of PCNA mediated stimulation of Fen 1, Lig I, and Pol δ activity (54,56). Recently it has been proposed that the 9-1-1 complex may substitute for PCNA during cellular stress (57). According to this hypothesis the 9-1-1 complex might act as a platform for repair proteins when the ability of PCNA to stimulate certain components of BER is reduced due to the binding to p21. Importantly, the activity of Pol β is not affected by PCNA but is strongly enhanced by the 9-1-1 complex (39).This is in contrast to the enzymatic activity of Pol δ, which requires PCNA but is not influenced by the 9-1-1 complex (39). These properties of the two pols suggest that p21 induced by DNA damage inhibits PCNA-dependent Pol δ activity, whereas Pol β remains unaffected, thus allowing LP-BER to occur (54). On the other hand, since the 9-1-1 complex is recruited to the sites of DNA damage where it associates with various DNA damage sensors and repair proteins it may well be that it functions as a targeted response stimulator independently of the PCNA level (57).

In conclusion, our results demonstrate for the first time that the 9-1-1 complex is directly involved in LP-BER. Under *in vitro* conditions we showed that there is a hierarchy of stimulation by the 9-1-1 complex in the BER repairsosome and the enhancement of LP-BER activity occurs due to the specific stimulation of the two early components of repair machinery namely APE 1 and Pol β. Importantly, our data reflect a detailed biochemical study performed *in vitro* and, for the first time, an investigation of the effect of the 9-1-1 in a complex system containing several proteins. Although this system is simplified compared with the *in vivo* situation, it provides a tool to give a better insight in understanding how the 9-1-1 complex stimulates the different steps of the LP-BER process. Moreover, we showed in this report that the 9-1-1 complex interacts *in vivo* with APE 1, as it has previously been shown for several other LP-BER enzymes (41, 43, 45). Taken together the data presented in this manuscript and the previous studies performed on LP-BER and the 9-1-1 complex, directly connect the DNA damage response with DNA repair, suggesting that the 9-1-1 complex might act as a core component of this vital connection.

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Conflict of interest statement. None declared.

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6.2. Interaction and co-localization of apurinic/apyrimidinic endonuclease 1 with the checkpoint clamp loader, Rad17-RF-C_{2-5} complex in human cells

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Interaction and colocalization of apurinic/apyrimidinic endonuclease 1 with the checkpoint clamp loader, Rad17-RF-C_{2-5} complex in human cells

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Abstract

In response to DNA damage cells activate checkpoint signaling pathways that block cell cycle progression, regulate different repair pathways and, in case of an extensive damage, trigger apoptosis. The checkpoint sliding- clamp complex, Rad9-Rad1-Hus1 and the checkpoint clamp loader, the Rad17-RF-C_{2-5} complex have been characterized as sensors of DNA damage. Recently, it has been shown that besides its role in checkpoint signaling pathways, the 9-1-1 complex plays also a direct role in DNA repair by stimulating and/or interacting with several components of the long-patch base excision repair (LP-BER) machinery namely apurinic/apyrimidinic endonuclease 1 (APE1), DNA polymerase β (Pol β), flap endonuclease 1 (Fen 1), DNA ligase I (Lig I) and the MutY homologue of *Schizosaccharomyces pombe.* In this work we show for the first time that APE1 interacts *in vivo* and *in vitro* with the checkpoint clamp loader, Rad17- RF-C_{2-5} complex, thus providing another link between DNA damage checkpoint and DNA repair.

Introduction

The mammalian apurinic/apirimidinic endonuclease/ redox-factor1 (APE1/Ref1) is a very abundant protein whose subcellular distribution depends on the cellular physiological statement (1). The expression of the APE/Ref-1 gene is inducible by oxidative stress and over-expressed APE1/Ref-1 protein protects cells against the genotoxic and cell killing effects of reactive oxygen species (ROS). APE1/Ref1 serves multiple cellular functions. Except of its role in base excision repair (BER), it is involved in oxidative DNA damage repair and stimulates the DNA binding activity of AP-1 (Fos, Jun) proteins as well as nuclear factor-κB (NF-κB), the polyoma virus enhancer-binding protein 2 (PEBP2), the early growth response-1 (Egr-1), Myb, members of the ATF/CREB family, the hypoxia inducible factor-1a (HIF-1a), the HIF-like factor (HLF), Pax-5 and Pax-8 (reviewed in (2)). APE1 possesses also the major AP-1 redox activity in cells and represents a novel redox component of signal transduction cascades that regulates eukaryotic gene expression. In addition APE1 has been implicated in the controlling of p53 activity through redox dependent and independent mechanisms. It has been also closely linked to apoptosis and altered levels of APE1 have been found in some cancers. Therefore APE1 appears to form a link between the BER pathways, cancer, and regulation of transcription factors, oxidative signaling and cell cycle control.

The cellular response to DNA damage consists of cell cycle checkpoints and DNA repair mechanisms that act in highly coordinated way to maintain genomic stability. The checkpoint response is based on the mechanism of signal transduction cascade, where sensor proteins detect a lesion in the double helix and stimulate several effectors through the activity of different protein kinases. This may lead to temporary cell cycle arrest, slowing down of DNA replication, changes in the cellular transcriptional program, chromatin remodeling, induction of DNA repair genes and, in some instances, apoptosis. (3,4). In mammalian cells, some of the major checkpoint proteins namely ataxia telangiectasia mutated protein (ATM), ATM-related protein (ATR), ATR interacting protein (ATRIP), Rad17, Rad9, Rad1 and Hus1, have been suggested to be involved in triggering DNA repair processes. Among those, the three human proteins Rad9, Hus1, and Rad1 (called the 9-1-1 complex) form a ring-shape

complex which shares sequence similarity with the homotrimeric clamp formed by proliferating cell nuclear antigen (PCNA). The 9-1-1 complex is loaded onto chromatin in response to different genotoxic stresses including alkylation, ultraviolet light, ionizing radiation, and replication inhibitors suggesting that the complex acts in cellular responses activated by many types of DNA damage. Moreover, the Rad17-RF-C_{2-5} complex has been characterized as a sensor of DNA damage and is targeted to the nucleus and damage sites following genotoxic stress (3,5-11). In addition, Rad17 shares homology with all five subunits of the heteropentameric replication factor C complex (RF-C), and it associates with the four small RF-C subunits in mammalian cells and budding yeast (12-20). Several studies showed that the 9-1-1 complex and Rad17-RF-C_{2-5} function as a clamp/clamp–loader pair, similarly to PCNA and RF-C (5,6,10,18,21). Moreover, the 9-1-1 complex, Rad17-RF-C_{2-5} and PCNA co-localize in foci formed upon DNA damage (5,22,23). The current model proposes that Rad17-RF-C_{2-5} would recognize DNA lesions, allowing the recruitment of the 9-1-1 complex to those sites, where it may facilitate the recruitment of the checkpoint effector kinase Chk1, subsequently phosphorylated by the ATR/ATM kinases (11,24-26). In human cells chromatin associations of Rad17 and ATR are largely independent suggesting that these proteins localize to the site of DNA damage independently. In addition, Rad17 is able to recruit the 9-1-1 complex even in the absence of ATR which indicates that Rad17 functions in parallel with ATR in damage detection (11). In response to DNA damage and replication inhibitors ATR phosphorylates Rad17 on Ser^{635} and Ser^{645} (27,28) and this phosphorylation is thought to be required for Rad17 mediated G2 arrest, preassembly via Chk1 (28). Importantly, Rad17 is not only important for ATR-mediated checkpoint, but is also essential for cell survival in mammals and other eukaryotes (cells lacking Rad17 exhibit acute chromosomal aberrations and undergo endoreduplication at high rate) (11,28,29). Interestingly, hRad17 is also phosphorylated on the same two serine residues during unperturbed S phase, suggesting a role of hRad17 during DNA replication (27). In support to this finding it has been shown that phosphorylated Rad17 co-localizes to sites of ongoing DNA replication where it associates with Pol e and the hMCM7 protein (30,31) which provides a link between the DNA damage checkpoint machinery and the replication apparatus.

Despite to their well documented roles in checkpoint sensing and signaling, several reports have implicated the 9-1-1 complex and Rad17-RF-$C_{2–5}$ in various DNA repair pathways (23,32-34). Moreover, recent investigations showed several possible connections between the human 9-1-1 complex and the long patch base excision repair (LP-BER) machinery (Table 1) (35-43) thus providing a direct link between DNA damage response and DNA repair. In this study we show for the first time that human APE1 interacts *in vitro* and *in vivo* not only with the checkpoint clamp, the 9-1-1 complex but also with the checkpoint clamp loader, the Rad17-RF-$C_{2–5}$ complex. Moreover, we demonstrate that APE1 and Rad17 co-localize in human cells and that this interaction is enhanced upon DNA damage. Overall, our results reveal another link between the DNA damage checkpoint response and the DNA repair machinery.

Materials and methods

Chemicals. Protein G agarose beads were from Amerscham Biosciences. Anti-Flag M2 affinity gel was from Sigma. All other reagents were of analytical grade and were purchased from Merck or Fluka.

Proteins and antibodies. Human APE1 was purified as previously described (44). Bovine serum albumin (BSA) was purchased from New England BioLabs. The human His-Rad9-1-1 complex and Flag-Rad17-RF-C complex were purified as described with modification (16). Briefly, 9-1-1- complex was isolated by co-expressing in Sf9 insect cells the three baculoviruses encoding the recombinant human Rad1, His-Rad9 and Hus1. The complex was subsequently purified using Ni-NTA Sepharose and Q Sepharose chromatography. The Flag-Rad17-RF-C_{2-5} complex was isolating by co-expressing the five baculoviruses encoding the recombinant human Flag-Rad17, p36, p37, His-p38, and p40 in Sf9 insect cells. The complex was subsequently purified using Ni-NTA Sepharose, anti-Flag agarose and Q Sepharose chromatography. A polyclonal antibody recognizing human Rad9 protein was kindly provided by R. Freire (Tenerife, Spain). A monoclonal antibody to human Rad9 protein was obtained from Alexis. A polyclonal antibody to hAPE1 (anti-Ref1, H300) and a polyclonal antibody to hRad17 were purchased from Santa Cruz Biotechnology.

Pull-down assays. For the Flag pull-downs, 50 µg of M2-Flag agarose beads were coated for 3 hours at 4°C with 100µg of BSA in pull-down buffer (40 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5mM $MgCl_2$, 10% glycerol (v/v), 0.05% (v/v) NP-40). After extensive washing, 5µg of the Flag-tagged Rad17- RF-C_{2-5} complex was added to the beads, following by incubation for 2 h at 4°C. After three series of an extensive washing, 5 µg of purified APE1 or 5 µg of the 9-1-1 complex were added to the beads and further incubated for 2 h at 4°C in pull-down buffer. For the negative controls, the Flag-Rad17-RF-C_{2-5} complex was omitted from the reaction and corresponding amounts of hAPE1 and the 9-1-1 complex were added to the beads. Next, the supernatant was removed, beads were washed five times in pull-down

buffer, and heated for 5 min at 95°C in Laemmli buffer. The co-precipitated proteins were analyzed by western blot using the corresponding antibodies according to established methods.

Whole cell extracts preparation. For preparing total cell extracts, 293T were treated with 1mM MMS or 1mM H_2O_2 for 1 hour, the media were replaced and cells were left to recover for additional 1 hour. Next, the cells were harvested and washed with PBS, followed by centrifugation. The cell pellet was subsequently lysed in cell lysis buffer (50mM Hepes-KOH, pH 7.5, 400mM NaCl, 1mM DTT, 2.5 mM $MgCl_2$, 20% glycerol (v/v), 0.5% (v/v) NP-40, 2µg /ml leupeptin, 1µg /ml bestatin, 1µg /ml pepstatin, 2mM PMSF, 10mM glycerophosphate, 1mM NaF, 10mM $Na_4P_2O_7$) for 15 minutes at 4°C. The cell lysate was subsequently centrifuged for 15 minutes at 10 000 rpm (Rotor FA45-30-11) and the supernatant was collected and kept as a total cell extract. The protein concentration in the extract was determined using the Bradford assay.

Co-immunoprecipitation experiments. For co-immunoprecipitations, Protein G sepharose beads (30ml per assay point) were coated for 3 hours at 4°C with 100µg of BSA in IP buffer (40mM Hepes-KOH, pH 7.5, 100mM NaCl, 8 mM $MgCl_2$, 2µg /ml leupeptin, 1µg /ml bestatin, 1µg /ml pepstatin, 2mM PMSF, 10mM glycerophosphate, 1mM NaF, 10mM $Na_4P_2O_7$) and subsequently incubated overnight with 2 µg of polyclonal anti-Rad9 or anti-Rad17 antibody in IP buffer. For the negative control, 2 µg the non-immunized rabbit IgG were incubated with the beads. Exponentially growing 293T cells (2x 10^7 per assay point) were washed in phosphate-buffered saline and lysed in lysis buffer (50mM Hepes-KOH, pH 7.9, 400mM NaCl, 8 mM $MgCl_2$, 10% glycerol (v/v), 0.5% NP-40 (v/v), 2µg /ml leupeptin, 1µg /ml bestatin, 1µg /ml pepstatin, 2mM PMSF, 10mM glycerophosphate, 1mM NaF, 10mM $Na_4P_2O_7$) for 10 min on ice. Cleared lysates (14 000 rpm, 10 min (Rotor FA45-30-11) 1mg of total cell extract were immunoprecipitated with the indicated rabbit polyclonal antibodies or the non-immunized rabbit IgG and protein G- Sepharose for 3 hours at 4°C. Immunoprecipitates were washed three times with IP buffer containing 0.05% (v/v) NP-40, the beads were incubated with 1mg of 293T total cell extract for 3 h at 4°C.

After incubation, the supernatant was removed and the beads were washed five times in IP buffer containing 0.05% (v/v) NP-40 and subsequently heated for 5 min at 95°C in Laemmli buffer. The co-immunoprecipitated proteins were analyzed by western blot using the corresponding antibodies according to established methods.

Immunofluorescence. Immunofluorescence experiments were performed using U2OS or HeLa cells grown on sterile glass cover slips. After treatment with 1mM MMS or 1mM H_2O_2 for 1 hour, the media were replaced and cells were left to recover for additional 1 hour. Next, the cells attached to cover slips were washed with PBS and fixed using 100% methanol (U2OS cells) or 4% parafalmaldehyde (HeLa cells), followed by blocking in 3% milk for 1 hour. Detection of hAPE1, hRad9 and hRad17 was performed using purified rabbit anti-hAPE1 antibody (1/300), mouse anti-Rad9 antibody (1/100) and mouse anti-hRad17 antibody (1/100), respectively. After overnight incubation with primary antibody, the cells were washed with PBS and subsequently incubated with monoclonal Texas Red-labelled (1/100) or polyclonal FITC-labelled (1/250) secondary antibodies, respectively, followed by washing with PBS and incubation with DAPI (Molecular Probes). Cells were observed with a Leica DMRB microscope equipped with a 100W HBO lamp for fluorescence. High-resolution pictures were taken with oil-immersion lenses (PL-FLUOTAR 40x-100x) and images were captured with a Leica DC 200 camera. Cells were viewed using Leica DC Viewer software and image merging was obtained using Adobe Photoshop Elements 2.0.

Results

APE1 co-localizes with the 9-1-1 complex after DNA damage*.* Previous studies showed that both APE1 and the 9-1-1 complex translocated to the nucleous in response to a variety of DNA-damaging agents (1,22,45). Moreover, we showed recently that both proteins could interact in untreated human cell extract (37).Therefore, we reason that APE1 as well as the 9-1-1 complex could be localized to the same sites of DNA damage and their interaction could be enhanced upon stress condition. To address whether genotoxic stress could stimulate the observed interaction the potential co-localization of APE1 and the 9-1-1 complex in the same nuclear foci upon DNA damage was investigated. Treatment with 1mM MMS or 1mM H_2O_2, was applied in order to introduce oxidative damage (Figure 1, panel E-H and I-L respectively). After 1 hour of treatment, media were replaced and cells were left to recover for additional 1 hour. Next media were removed and cells were fixed and stained as described in Materials and Methods. As show in Figure 1 (panel A and B) in untreated cells both Rad9 and APE1 and appeared to be homogenously distributed throughout the nucleus. Upon exposure to 1mM MMS or 1mM H_2O_2, a clear redistribution of Rad9 and APE1 proteins to form discrete nuclear foci occurred (Figure 1, Panel E-L). Some extent of co-localization of these foci was observed, as indicated in Figure 1A, panels H and L, respectively. Such limited degree of co-localization was probably reflecting the fact that both APE1 and Rad9 serve multiple cellular functions and interact with numerous protein partners in cells. Taken together, these results additionally support our previous finding that APE1 and the 9-1-1 complex interact *in vivo* and indicate, that in response to genotoxic stress both the 9-1-1 complex and APE1 are translocated to the sites of DNA damage where the 9-1-1 complex could recruit and eventually stimulate endonuclease activity of APE1 in BER.

APE1 interacts in vivo with the Rad17-RF-C_{2-5} complex. We have previously shown that APE1 could be co -immunoprecipitated with the 9-1-1 complex using total cell lysates prepared from untreated, human 293T cells (37). Our next aim was to determine if the amount of APE1 co-immunoprecipitated with anti- Rad9 antibody

could be increased upon genotoxic stress condition. To answer this question, treatment with 1mM MMS or 1mM H_2O_2 (Figure 2, panel B) was applied. After 1 hour of treatment, media were replaced and cells were left to recover for additional 1 hour. A total cell extract prepared from 293T was subsequently incubated with anti-Rad9-conjugated Protein-G Sepharose beads and precipitated proteins were detected by western blot using respective antibodies. Upon the treatment with the two damaging agents, the level of APE1 co-immunoprecipitated with the 9-1-1 complex, remained unchanged (Figure 2, panel B, lower). Additionally it was also confirmed that Rad17 was present in the fraction of co-immunoprecipitated with anti-Rad9 antibody proteins (Figure 2, panel B, upper). The next question was to determine whether the Rad17-RF-C_{2-5} complex could also interact with APE1 directly. To test this possibility, similar co-immunoprecipitation experiments using anti-Rad17-conjugated Protein G Sepharose and total cell extract from human 293T cells were performed. Indeed, APE1 was detectable in the fraction of co-immunoprecipitated precipitated proteins, whereas it was not detectable in the negative control performed with non-immunized rabbit IgG (Figure 2C, bottom). To test if various forms of DNA damage could stimulate *in vivo* interaction between APE1 and Rad17-RF-C_{2-5} complex, the same treatment as described above was used. Similarly to the results obtained from the studies on APE1/9-1-1 complex interaction, also in this case, DNA damage did not cause any significant changes in the amount of APE1 co-immunoprecipitated with anti-Rad9 antibody. However, we cannot exclude the possibility that the ionic strength of the extraction buffer (400mM NaCl) we used in the co-immunoprecipitation study, was too high and harsh to observe very transient and subtle differences in the protein level especially also the level of Rad9 protein co-immunoprecipitated with anti- Rad17 antibody remained unchanged upon applied treatment.

APE1 and the Rad17-RF-C_{2-5} complex co-localize upon DNA damage. Next we determined if APE1 and Rad17 could also co-localize to the same nuclear foci upon the treatment with DNA damaging agents. As shown in Figure 1 (panel A and B) in untreated cells both Rad17 and APE1 show a rather homogenous distribution throughout the nucleus. Upon exposure to 1mM MMS or 1mM H_2O_2, discrete nuclear

foci of both proteins occurred (Figure 1, Panel E-L), which co-localized to some extent (Figure 1A, panels H and L respectively). In agreement with the results described above, such limited degree of co-localization was probably due to the fact that the Rad17-RF-C_{2-5} complex, similarly to APE1 and the 9-1-1 complex, interact with various protein partners and functions in multiple biological processes in cell.

APE1 and the Rad17-RF-C_{2-5} complex interact in vitro. Because the 9-1-1 complex was shown to interact physically and functionally with APE1, we considered the possibility that the interaction between APE1, and the Rad17-RF-C_{2-5} complex was mediated by the independent binding of both protein to the 9-1-1 complex. To test this possibility we performed pulldown experiments using purified, Flag- tagged Rad17-RF-C_{2-5} complex and APE1 (Figure 4, line 2 and 3). Incubation of Flag-Rad17-RF-C_{2-5}, previously bound to anti-Flag agarose, with purified APE1, revealed that the two proteins physically associated as APE1 was detected in the pulled-down fraction (Figure 4, lane 4), but not in the fraction where the checkpoint clamp loader was omitted from the reaction (Figure 4, line 6). As an additional control for the proper condition of the experiment purified 9-1-1 complex was tested (Figure 4, line 1). In this pulldown, the 9-1-1-complex was also co-precipitated with the Rad17-RF-C_{2-5} complex but was not present in the pulled-down fraction when incubated with the beads alone. In summary, these results demonstrated for the first time a direct physical interaction of the Rad17-RF-C_{2-5} complex with APE1.

Discussion

The two sensors proteins, the checkpoint clamp, 9-1-1 complex and its clamp loader, Rad17-RF-C_{2-5} complex are the key components of the DNA damage checkpoint response. Moreover, recent investigations have established a link between the checkpoint response and the BER machinery indicating the 9-1-1 complex as a core component of this connection (Table 1). Previously we reported the physical and functional interaction between the 9-1-1 complex and APE1 endonuclease. In this work we further analyze the physical interaction between the two proteins and provide the evidence that upon exposure to genotoxic stress, both APE1 and Rad9 co-localize at the discrete nuclear foci. The limited extent of the co-localization of these foci was probably due to the fact that both APE1 and Rad9 possess multiple cellular functions and interact with numerous protein partners in the cell. Thus, it is not surprising, that only certain nuclear fractions of both proteins, presumably involved in the same repair pathway, co-localized. Taken together, these results additionally support our previous findings that APE1 and the 9-1-1 complex interact *in vivo* and indicate, that in response to genotoxic stress both the 9-1-1 complex and APE1 are translocated to the sites of DNA damage where the 9-1-1 complex could recruit and eventually stimulate the endonuclease activity of APE1 in BER. However our efforts to demonstrate that DNA damage stimulates the interaction between the 9-1-1 and APE1 also in human total cell extract brought no answer. Nevertheless, we cannot exclude the possibility that the ionic strength of the extraction buffer (400mM NaCl) used in the co-immunoprecipitation experiment, interfered with very transient and subtle differences in the protein level that occur directly onto chromatin.

Previously we showed that *in vitro,* the loading of the 9-1-1 complex is not absolutely required for the stimulation of APE1 activity and we proposed that the observed stimulatory effect could result from protein-protein that does not involve the encircling of the DNA substrates (37). However, *in vivo* only the loading of the 9-1-1 complex onto chromatin by the Rad17-RF-C_{2-5} complex leads to the local increase of its concentration at the damage sites, where it functions in DNA damage signaling and most probably, in DNA repair. Interestingly, we present the evidence that the checkpoint clamp loader, Rad17- RF-C_{2-5} complex interacts with APE1 under in *vivo*

and *in vitro* condition. In response to DNA damage APE1 and Rad17 redistribute in the nucleous in order to form discrete nuclear foci that appeared to co-localize to some extent. In agreement with findings described above about APE1/Rad9 co-localization, also in this case the limited number of co-localizing foci was expectable. Moreover, co-immunoprecipitation experiments further confirmed that the checkpoint clamp loader interacts with APE1 in total cell lysates prepared from human cells. In addition we could confirm the presence of the 9-1-1 complex in Rad17 immunoprecipitates suggesting that the two complexes and APE1 may associate to from a high molecular weight complex. However, we cannot rule out the possibility that the observed co-immunoprecipitation of APE1 and Rad9 with the Rad17 antibody as well as APE1 and Rad17 with Rad9 anybody may result from independent protein-protein interaction and the presence of all three proteins in the immunoprecipitates reflects the sequential, indirect mechanisms of interaction. Importantly, we could also confirm that the Rad17- RF-C_{2-5} complex directly associates with APE1 as revealed by pulldown experiments with purified human proteins. Taken together, these findings suggest that the checkpoint clamp loader, Rad17- RF-C_{2-5} complex directly participate in BER thus providing another link between the DNA damage response and DNA repair. In support to this hypothesis, a conserved interaction between the Rad17- RF-C_{2-5} and another BER component, Lig I has recently been recently described (46). The complex interacted with Lig I *in vivo* and *in vitro* and this interaction occurred via N-terminal domain of its large subunit, Rad17. Interestingly, similar interaction between Lig I and replicative clamp loader, RF-C have been observed (47). This interaction was also mediated via the large subunit of RF-C, p140 although the two complexes were shown to bind to different regions on Lig1. Moreover, RF-C exerts an inhibitory effect on Lig I activity whereas Rad17-RF-C_{2-5} stimulates the joining process. Similar results were obtained with the homologous *Saccharomyces cerevisiae* proteins indicating that the interaction between the replicative Lig and checkpoint clamp is conserved in eukaryotes. Notably, the Rad17-RF-C_{2-5} complex was shown to preferentially interact and stimulate dephosphorylated form of Lig I. Taken together, it seems possible that the interaction between RF-C and Lig I may be involved in the unloading of PCNA after the joining of adjacent Okazaki fragments (47,48) whereas Lig I and Rad17-RF-C_{2-5},

and as shown in this report, APE1 and Rad17- RF-C_{2-5} may play a similar role in unloading the Rad9-Rad1-Hus1 complex, thereby switching off the checkpoint when the damage is removed and restarting the replication process. However, it would be interesting to determine the possible domain/domains on APE1, involved in the interaction with the checkpoint clamp/clam loader pair and establish the correlation between the replicative clamp/clamp loader complex. Further more it will be necessary to examine the influence the Rad17-RF-C_{2-5} complex on those components of the BER machinery, that are already known to interact physically and functionally with the 9-1-1 complex. Overall, the data presented in this work, together with previous reports indirectly connect the DNA damage response with DNA repair, placing the checkpoint clamp, 9-1-1 complex and its clamp loader, the Rad17-RF-C_{2-5} complex at the crossroads of these interactions.

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Table1. Functional and physical interactions between the 9-1-1 complex and Rad17-RF-C_{2-5} complex and components of the LP-BER machinery

Checkpoint sensor	LP-BER component	Functional and physical interaction	Reference
9-1-1	MutY DNA glycosylase	Stimulation of glycosylase activity *In vitro* and *vivo* interaction	(35,39)
9-1-1	APE1	Stimulation of AP endonuclease activity *In vitro* and *vivo interaction*	(37)
Rad17-RF-C_{2-5}	APE1	*In vitro* and *vivo* interaction	Present study
9-1-1	Pol β	Stimulation of polymerase and strand displacement activities *In vitro* and *vivo* interaction	(41)
9-1-1	Fen 1	Stimulation of endo- and exonuclease activities *In vitro* and *vivo* interaction	(36,42)
9-1-1/Rad17-RF-C_{2-5}	Lig I	Stimulation of ligase activity *In vitro* and *vivo* interaction	(40,43,46)

Figure legends

Figure 1. Co-localization of hAPE1 with hRad9 following oxidative stress. Cells were either left untreated *(NT),* treated with 1mM MMS *(MMS)* or 1mM H_2O_2 *(H_2O_2)* for 1 hr and then left to recover for additional 1hr. The cells attached to cover slips were washed with PBS, fixed and incubated with primary and secondary antibodies following by the visualization with fluorescent microscopy. (A) Human U2OS cells were stained with anti-Rad9 antibody (green, subpanels A,E,I) and anti-APE1 antibody (red, subpanels B,F,J). Subpanels C,G,K are the DAPI (4',6'-diamidino-2phenylindole)-stained nuclei. Subpanel D,H,L are the merged images of the anti-Rad9 and anti-APE1 stained cells. (**B)** As **(A)** but with HeLa cells fixed with 4% parafalmaldehyde.

Figure 2. *In vivo* physical interaction between APE1 and the 9-1-1 complex. Exponentially growing 293T were either left untreated *(NT)* or treated with 1mM MMS *(MMS)* or 1mM H_2O_2 *(H_2O_2)* for 1 hr followed by 1hr of recovery. Total cell lysates were prepared and used for co-immunoprecipitation (IP) assays with anti-Rad9 (panel B), anti-Rad17 (*panel C*) antibodies or normal rabbit IgG (*panel D*) as described in Materials and Methods. Proteins from the immunoprecipitates were detected by Western blotting using anti-Rad17 antibody *(upper panel)*, anti-Rad9 antibody *(middle panel)* and anti-APE1 antibody. As control, 5% of the total volumes of the whole cellular lysates used for the co-IP reactions were also included (*Input, Panel A*). Data are representative of three independent experiments.

Figure 3. Co-localization of hAPE1 with hRad17 following oxidative stress. HeLa cells were either left untreated *(NT),* treated with 1mM MMS *(MMS)* or 1mM H_2O_2 *(H_2O_2)* for 1 hr, followed by 1hr of recovery. The cells attached to cover slips were washed with PBS, fixed with 4% parafalmaldehyde and incubated with primary and secondary antibodies and visualized with fluorescent microscopy. HeLa cells were stained with anti-Rad17 antibody (green, subpanels A,E,I) and anti-APE1 antibody (red, subpanels B,F,J). Subpanels C,G,K are the DAPI (4',6'-diamidino-

2phenylindole)-stained nuclei. Subpanel D,H,L are the merged images of the anti-Rad9 and anti-APE1 stained cells.

Figure 4. *In vitro* physical interaction between APE1 and the Rad17-RF-C_{2-5} complex . FLAG-pulldown experiments were performed in the presence of purified FLAG-Rad17-RF-C (10µg), previously immobilized on FLAG agarose beads, and APE1 (5µg) (lane 4) or the 9-1-1 (5µg) complex as a positive control (lane 5). As negative controls APE1 (lane 6) or the 9-1-1 complex (lane 7) were incubated with FLAG agarose beads alone. The presence of co-precipitated proteins was determined by SDS-PAGE followed by Western blot analysis using anti-Rad17 antibody (upper panel), anti-Rad9 antibody (middle panel) and anti-APE1 antibody (lower panel). 5% of purified proteins used for pulldown assay was loaded as control (lane 1-3, the 9-1-1 complex, APE1, Rad17-RF-C_{2-5} respectively. Data are representative of three independent experiments.

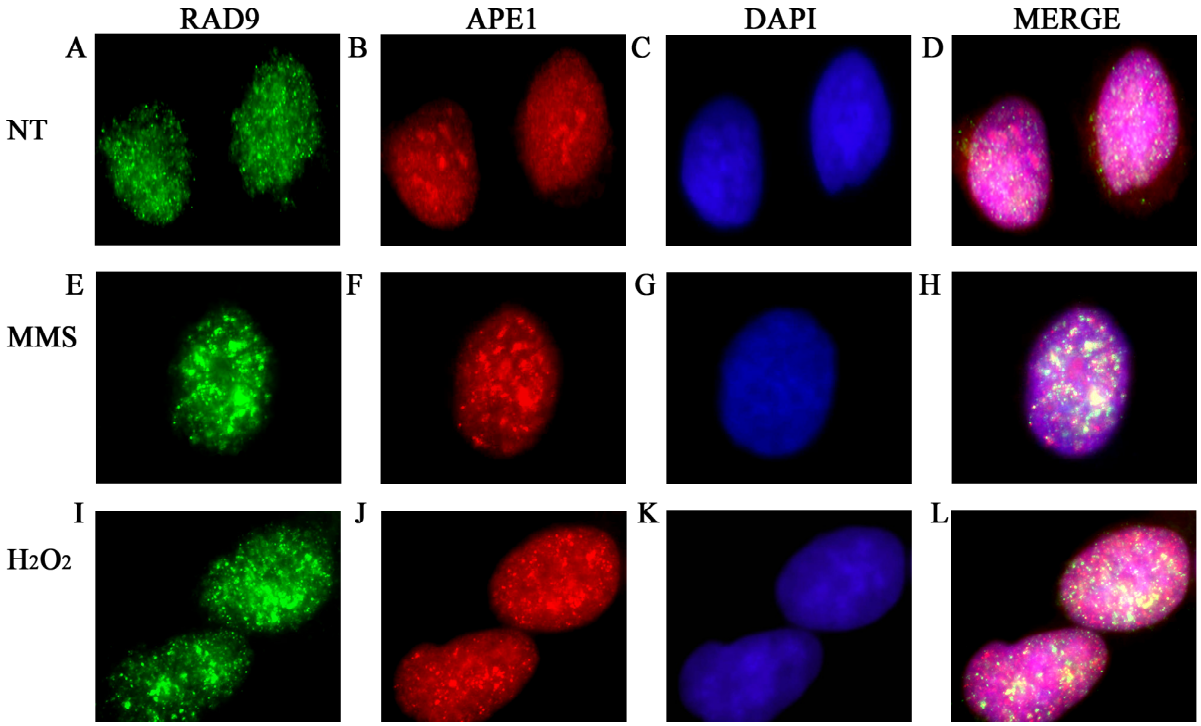

Fig. 1a, Gembka et al.

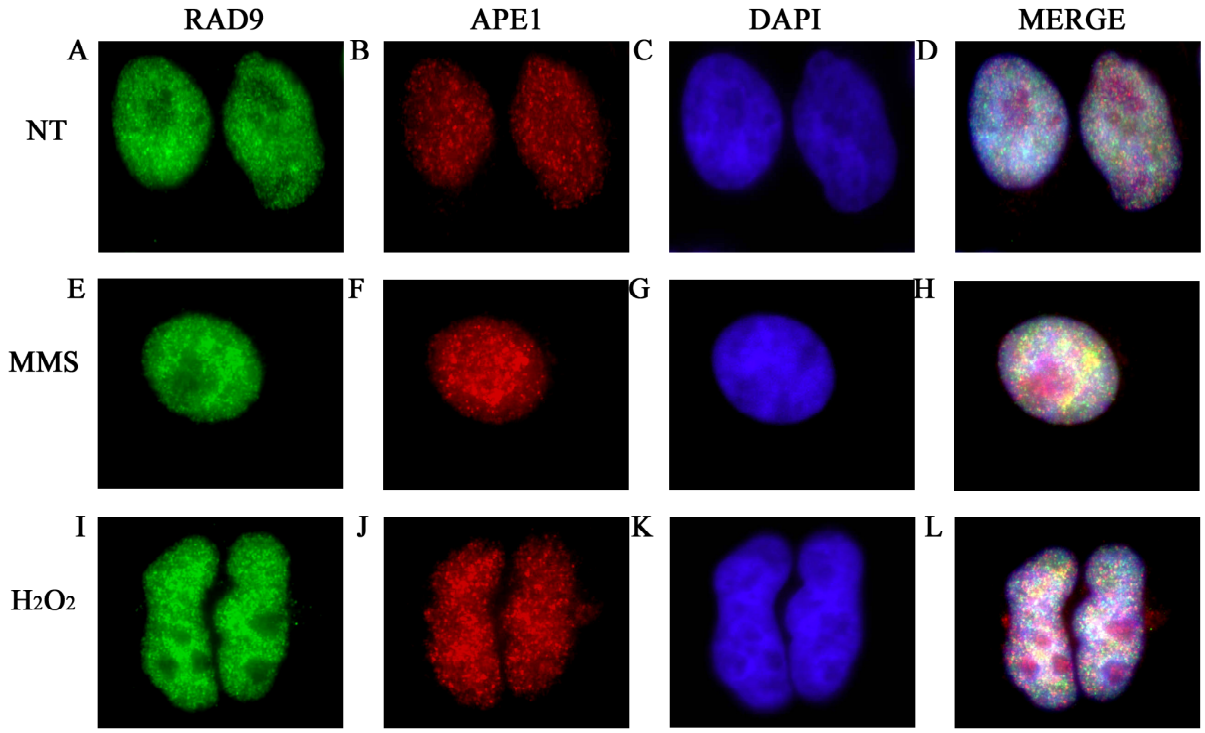

Fig. 1b, Gembka et al.

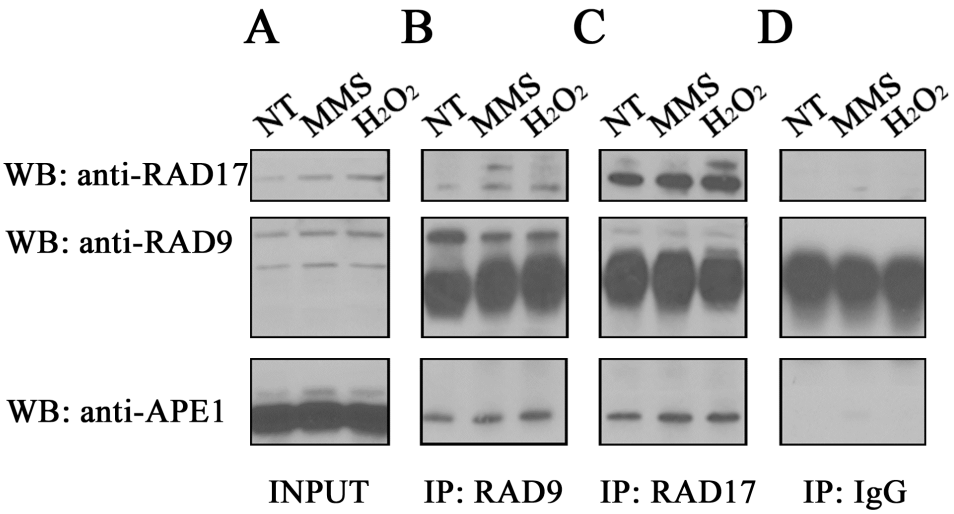

Fig. 2, Gembka et al.

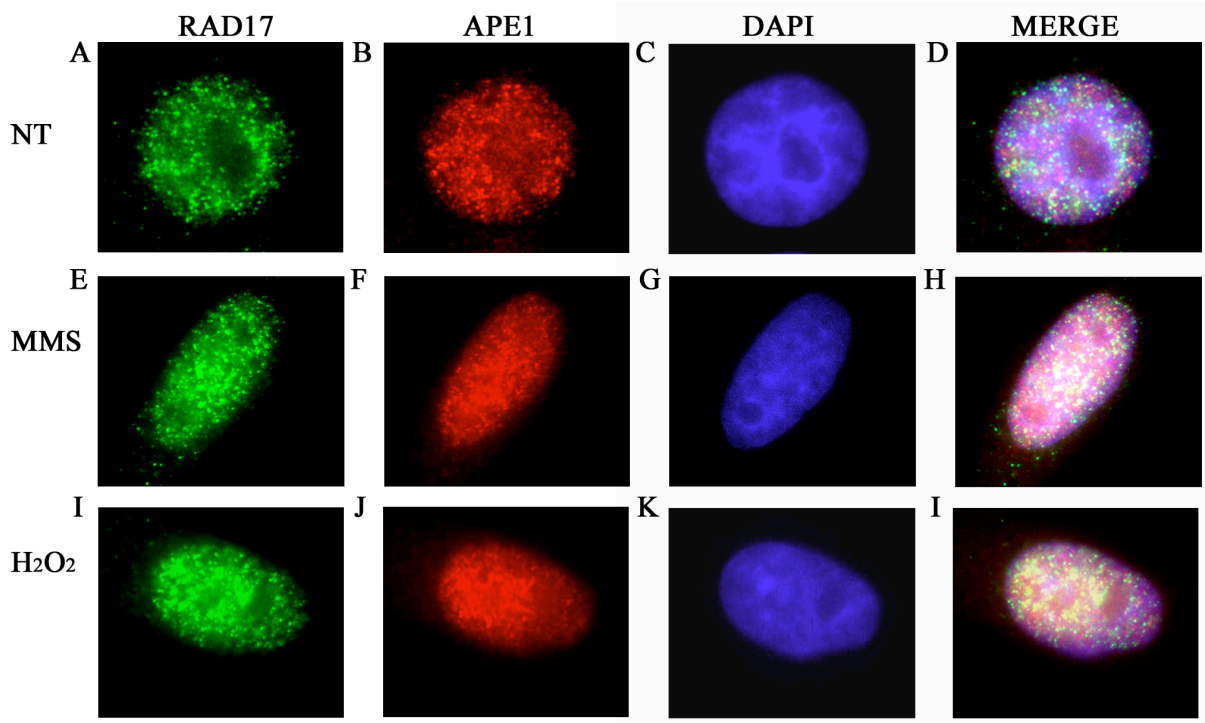

Fig. 3, Gembka et al.

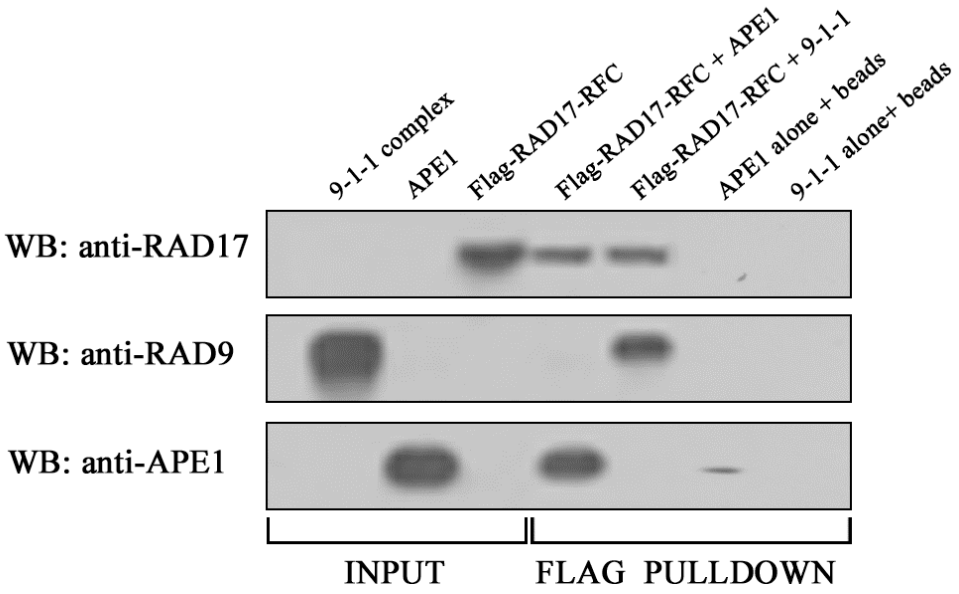

Fig. 4, Gembka et al.

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7. CONCLUSIONS AND PERSPECTIVES

Rad17, Rad9, Rad1, Hus1 are members of Rad family of checkpoint proteins that are required for cell cycle arrest in response to DNA damage or inhibition of DNA synthesis. Three of these proteins, Rad9, Rad1 and Hus1 form a ring-like complex (called the 9-1-1 complex), which structurally resembles the homotrimeric clamp formed by proliferating cell nuclear antigen (PCNA), whereas Rad17 associates with the four small subunits of the heteropentameric RF-C complex in a manner similar to the RF-C. The two complexes have been characterized as sensors of DNA damage. The current model suggests that following genotoxic stress, Rad17- RF-$C_{2–5}$ can recognize DNA lesions, allowing the recruitment of the 9-1-1 complex to those sites. ATM and ATR kinases are recruited simultaneously to the same sites of DNA damage but in a 9-1-1 complex and Rad17-RF-$C_{2–5}$ complex independent manner. However, a growing number of evidence indicates that the checkpoint clamp, 9-1-1 complex and the checkpoint clamp loader, Rad17- RF-$C_{2–5}$ complex, besides their functions in the DNA damage checkpoints and signaling pathways play also more direct roles in various DNA repair pathways including nucleotide excision repair, translesion synthesis, homologous recombinantional repair and base excision repair.

This thesis work describes the novel interactions of the 9-1-1 complex and the Rad17-RF-C_{2-5} complex with the early component of BER machinery, APE1. The 9-1-1 complex has been shown to interact *in vitro* and *in vivo* with APE1 and stimulate its AP-endonuclease activity in a single enzyme assay as well as in the reconstituted LP-BER *in vitro.* Further analysis has demonstrated that the 9-1-1 complex could also stimulate LP-BER *in vitro* through the stimulation of the strand displacement activity of Pol β . However, under the same conditions, the checkpoint clamp could not influence the enzymatic activities of Fen 1 and Lig I indicating that in a reconstituted LP-BER *in vitro* system, a hierarchy of interactions between the 9-1-1 complex and the components of the LP-BER machinery exists. Moreover, human APE1 has been shown to interact *in vitro* and *in vivo* not only with the checkpoint clamp, the 9-1-1 complex but also with the checkpoint clamp loader, the Rad17-RF-$C_{2–5}$ complex. Both checkpoint sensors co-localized with APE1 in human cells and this interaction was enhanced upon DNA damage. Taken together, these data

supported by the previous studies performed on BER components and the 9-1-1 complex, suggest a model where the 9-1-1 complex and the Rad17- RF-C_{2-5} complex might recruit different BER factors to the damage sites thus providing a structural framework for DNA repair machinery. Moreover, once located at the lesion, the checkpoint clamp/clamp loader pair might exert their stimulatory effect on the enzymatic activities of BER proteins via simple protein-protein interaction. However, the molecular details underlying the mechanism of APE 1 stimulation by the 9-1-1 complex remain to be elucidated. Furthermore, additional *in vitro* analysis of the Rad17-RF-C_{2-5} involvement in the LP-BER machinery could provide exciting answers about the functional interactions of the checkpoint clamp loader with APE1 and other components of LP-BER. Another open question is to identify the site(s) on APE1, involved in the interaction with the checkpoint clamp/clamp loader pair. Previously it has been shown that another components of LP-BER pathway, namely Fen 1 and Lig I interact with the 9-1-1 complex via different motifs the ones utilized by PCNA. Moreover, recent studies have revealed that the Rad17-RF-C_{2-5} complex physically and functionally interacts with Lig I although via different interaction domain than RF-C. Importantly, also the effect the two clamp loaders exerted on Lig I activity differed. Thus, it would be important to establish how the binding of the checkpoint clamp/clamp loader pair to APE1 interferes with the similar interaction between the endonuclease and the replicative clamp/clamp loader complex. Based on the interactions between the 9-1-1 complex and the BER proteins that are also known to interact with PCNA, a model has emerged where the heterotrimeric clamp and presumably Rad17-RF-C_{2-5} could replace PCNA and RF-C in certain DNA excision repair pathways during cellular stress. This model could also provide the answers how DNA repair pathways can operate when DNA replication is inhibited and PCNA is bound by p21. In summary, the results presented in this thesis directly connect the DNA damage response with DNA repair, placing the 9-1-1 complex and possibly the Rad17-RF-C_{2-5} complex at the head of these connections.

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Curriculum Vitae

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Outstanding qualities

Project Management: Responsible for managing scientific projects including financial planning and budget control. Developed ideas to improve several techniques used in the laboratory. Set up processes and implemented them, adapting taken actions to available resources, both financial and technological. Member of the Organizing Committee of two scientific conferences, participated in all stages of the event organization: logistics, scientific content, membership and task management.

Teamwork and people management: Excellent in working independently and at the same time a team player in group projects. Supervised the work of younger graduate students. Great motivator and coach of the junior staff and master students.

Communication skills: Developed strong interpersonal skills while working in multicultural and multilingual scientific environment. Able to communicate research findings in an understandable and effective way. Co-author of several publications and a research grant proposal with strong writing and analytical skills as well as profound ability to use available resources, including several data bases and scientific internet portals.

Professional experience

2004-2007 **Institute of Veterinary Biochemistry and Molecular Biology,**
University of Zürich, Switzerland
Position held: PhD student

- Conceptually planned and executed two research projects "Physical and functional interactions between checkpoint sensors and components of the base excision repair machinery" and "Interaction and co-localization of apurinic/apyrimidinic endonuclease 1 with the checkpoint clamp loader, Rad17-RFC complex in human cells"
- Introduced novel molecular techniques previously not applied in the laboratory
- Set up external and internal collaborations
- Planned and supervised the work of graduates students
- Wrote scientific publications and reports
- Prepared presentations and posters for international and local conferences
- Arranged meetings and scientific gatherings
- In charge of lab radiation safety compliance

2003-2004 **Division of Gynecology, Department of Obstetrics and Gynecology**
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- Assisted in the research project "Analysis of the Ciliary Motility for Detection of Hormone and Hormone-like Effects on Cilia of the Respiratory Tract and of the Oviduct"
- Set up external collaborations
- Attended international and local conferences and meetings

1998-2003 **Department of Molecular Virology**
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- Assisted in the project design to investigate the possible role of migratory birds in the spreading of highly pathogenic avian influenza
- Fully executed the research project "Expression of Nucleoprotein Gene of Influenza A Virus in the Baculovirus Expression System"
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Education

2004-2007 **PhD in Molecular Biology (02.11.2007)**
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Biochemistry	Above average hands on knowledge of proteins expression (bacterial, baculovirus, and mammalian expression systems) and purification (ÄKTA Systems); protein-protein interaction and characterization (*in vivo* and *in vitro* methods), enzymatic assays, antibody generation and purification
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Computer	In-depth experience with Macintosh and Windows operating systems and standard software packages such as Word, Excel, PowerPoint, Photoshop and Acrobat

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Sport, martial arts (Aikido, Iaido), hiking, reading, working with dogs, traveling

Scientific publications

- "The checkpoint clamp, Rad9-Rad1-Hus1 complex preferentially stimulates the activity of apurinic/apyrimidinic endonuclease 1 and DNA polymerase β in long patch base excision repair". Agnieszka Gembka, Magali Toueille, Ekaterina Smirnova, Rainer Poltz, Elena Ferrari, Giuseppe Villani and Ulrich Hübscher; Nucleic Acid Research (2007) 35(8): 2596-608
- "The checkpoint sensors, the 9-1-1 complex and the Rad17-RFC$_{2-5}$ complex: At the crossroad of DNA damage checkpoints and DNA repair". Agnieszka Corsten- Gembka, Ekaterina Smirnova, Ulrich Hübscher, Invited review: DNA Replication, Repair and Recombination at the Crossroad Between Genome Integrity and Genomic Instability: a Biochemical Perspective (2007), ISBN 978-81-308-0253-4
- "Interaction and co-localization of apurinic/apyrimidinic endonuclease 1 with the checkpoint clamp loader, Rad17-RF-C$_{2-5}$ complex in human cells". Agnieszka Corsten-Gembka, Rebecca Buob, and Ulrich Hübscher*. Manuscript in preparation

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